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THE RELATIVE THICKNESS OF MOUSE SKINS TREATED WITH SULFHYDRYL, DISULFOXIDE AND DIBENZANTHRACENE

By

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(*Received June 19, 1937*)

Sulfhydryl compounds applied to mouse, rat, guinea pig and human skin cause hyperplasia of epithelium and subsequent increase in differentiation and organization of the cells (Hammett, '31, Reimann, '30, '31, Reimann and Hankele, '34). 1:2:5:6-dibenzanthracene also causes hyperplasia, but orderly differentiation and organization are disturbed. It is not known whether its effects are exerted primarily on the proliferative or on the succeeding phases of differentiation and organization, although experiments in an animal in which these phases can be well separated, seem to show that it is the proliferative mechanism which is primarily involved (Reimann and Hammett, '35). Since, however, cancer is produced by 1:2:5:6-dibenzanthracene, differentiation and organization must also be involved.

Cystine-disulfoxide, a partially oxidized derivative of the sulfhydryl cysteine (Toennies and Lavine, '36), inhibits the rate of cell multiplication in several species and under various experimental circumstances (Lavine, '35, Reimann and Hammett, '36).

Furthermore, a sulfhydryl (thiocresol) inhibits carcinogenesis by 1:2:5:6-dibenzanthracene (Reimann and Hall, '36) and a future publication will show that cystine disulfoxide has the same property.

To obtain quantitative data on the relative effects of thiocresol, 1:2:5:6-dibenzanthracene and cystine disulfoxide on the thickness of mouse skins, i.e., its proliferation, the following experiments were performed.

EXPERIMENTAL PROCEDURES

Thiocresol 0.5% in white petrolatum was rubbed daily into the skins of the right ears and over the backs of ten mice. 1:2:5:6-dibenzanthracene 0.3% in white petrolatum was used similarly in ten other mice. 0.5% cystinedisulfoxide in white petrolatum was rubbed daily into the same places of ten other mice. At the end of three weeks 18 (applications) the ears and slices of skins were

	No. of Specimens	Total Lengths	Total Areas	Area per 100 mm. length	Ratios
Controls	200	181 mm.	53.4 sq. mm.	29.4 sq. mm.	1
Thiocresol treated	200	161 mm.	143.6 sq. mm.	89.1 sq. mm.	3
Dibenzanthracene treated	200	245 mm.	106.7 sq. mm.	43.5 sq. mm.	1.5
Disulfoxide treated	200	203 mm.	72.3 sq. mm.	35.6 sq. mm.	1 2

* Aided by a grant from Mrs. L. Elizabeth Nax. Philadelphia.

removed, fixed in Sousa's solution, embedded in paraffin, cut at 7 micra and stained with hematoxylin and eosin. The sections were projected from an Edinger apparatus, the epithelium was traced, the length measured and a planimeter used to measure the area enclosed by the tracing. The magnification was set at ×200. The total length of skins measured and the areas covered are shown in the following table. It is obviously a measure of both numbers and sizes of cells.

DISCUSSION

Except for the skins treated with cystine disulfoxide, nothing need be added to previous descriptions as far as qualitative changes are concerned (Hammett, '31, Reimann, '30, '31, Reimann and Hall, '36, Reimann and Hankele, '34). The thiocresol-treated skins showed the same increase in number and enhancement of differentiation and organization of the epithelial cells; the dibenzanthracene-treated skins showed increase in number and mild disorientation. A very slight increase in number was discernible in the disulfoxide-treated skins; cell counts of 97 sections of each over lengths of 9 mm. showed 1810 cells in normal control and 2070 cells in the experimental sections. There was no increase in their degrees of differentiation and organization.

Since cystines disulfoxide, as a partially oxidized sulfhydryl derivative, inhibited the rate of proliferation in Obelia and the rate of growth of spontaneous mammary carcinomas of mice, and since Voegtlin found it inhibited the rate of multiplication of amebae, it was thought that the skins of mice treated with this compound would be thinner. The cells of the skin are, of course, active mitotically at all times; this replaces cells lost through wear and tear. But, as the table shows, there was no decrease in thickness. In the test tube, decomposition of cystines disulfoxide proceeds as follows.

$$2\ R(SO)_2R + 2H_2O \rightleftarrows 2RSOH + 2\ RSO_2H$$
$$\Updownarrow$$
$$RSH + RSO_2H$$

Thus sulfhydryl is formed which may be appropriated by some other group, or may in turn react with another molecule of cystine disulfoxide to form cystine as follows:

$$R\text{–}SH + R\text{–}(SO)_2\text{–}R \rightleftarrows R\text{–}S\text{–}S\text{–}R + RSO_2H.$$

The fate of cystine disulfoxide when ingested by mouth and sundry other biological data are being studied (Medes, Bennett, '37), but nothing is known of its fate when applied to skin. Perhaps minute amounts of sulfhydryl are liberated there. Meneghetti ('35) found colloidal sulphur injected intravenously into rats, guinea pigs and chickens was reduced to H_2S; RSOH also probably can be. Disulfoxide has a high acidity; this in itself tends to increase the reducing trend; perhaps it also helps thicken the skin by edema. Further speculation seems unwarranted at present.

SUMMARY

The skins of the back and ears of three groups of equal numbers of mice (10) were rubbed daily for 3 weeks with 0.5% thiocresol, 0.3% dibenzanthracene and 0.5% cystine disulfoxide in white petrolatum. The thicknesses of these skins were then measured in microscopic preparations. If the normal is taken as 1, the thickness of disulfoxide treated was 1.2, of dibenxanthracene 1.5 and of thiocresol 3. Numbers of cells, and degrees of differentiation and organization were enhanced in both thiocresol and dibenzanthracene treated skins, but much more in the former, while in the latter there was disorientation of the basal layer even in three weeks. No qualitative differences between normal and disulfoxide skins were detected.

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MECHANICAL FORCES AND TORQUE ON AN ELLIPSOID IN DIFFUSION FIELDS IN CONNECTION WITH THE PROBLEM OF ORIENTATION OF COLLOIDAL MICELLAE IN BIOLOGICAL SYSTEMS

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(*Received Oct. 26, 1937*)

Diffusion problems involving ellipsoidal boundaries are of considerable interest in mathematical biophysics, both in connection with cellular behavior and in the molecular theory of the physical constants of the cell. The biophysics of cellular multiplication and growth is based fundamentally on the study of diffusion fields (Rashevsky, '36a, b) which hitherto however have been considered only for spherical shapes. Many non-spherical cells are themselves representable to a good approximation by ellipsoids; while an ellipsoidal model for colloidal micellae dates back at least to the work of Gans ('12, '15, '20) on the optical properties of colloidal solutions. Suggested applications of this nature will be discussed more fully in the latter parts of this paper. At first we confine ourselves to the statement and solution of the mathematical problem, which may conceivably find application in connections not considered here, in either biological or non-biological studies.

The mathematical problem falls into two stages: to determine the modification of an original field by the ellipsoid; and then to calculate the force and torque on the basis of some assumed relation between concentration and pressure.

I: IMAGE FIELD

Given an original concentration field $c_1(x, y, z)$, we are to determine an image field c_2 which satisfies

(1)
a) $\nabla^2 c_2 = 0$ outside of the ellipsoid surface S
b) c_2 tends to zero at infinite distance from S
c) The normal derivatives of the modified field, $c = c_1 + c_2$, is zero everywhere on S.

These conditions specify an image field of the type familiar in hydrodynamics, rather than the kind used in electrostatics. They suffice to define c_2 uniquely.

The calculations will be given only for a prolate ellipsoid of revolution; they are essentially similar in the oblate case. An appropriate system of coordinates is given by (Mac Robert, '27)

(2)
$$x = (r^2 - f^2)^{1/2} \sin\theta \cos\phi$$
$$y = (r^2 - f^2)^{1/2} \sin\theta \sin\phi$$
$$z = r \cos\theta$$

where x, y, z are ordinary rectangular coordinates. The coordinate surfaces are confocal ellipsoids with semi-major axis r and foci at $(0, 0, \pm f)$; two-sheeted hyperboloids of revolution whose asymptotes are prescribed by the angle θ, which is the ordinary "eccentric angle" of the ellipsoids, ranging from 0 to π; and planes through the origin, normal to the x-y plane, prescribed by the angle of revolution ϕ, which has the range $0-2\pi$. This is a degenerate form of a complete ellipsoidal system, where the one-sheeted hyperboloids constituting the third set of coordinate surfaces have become a set of planes; it passes into an ordinary system of spherical coordinates for $f=0$.

In this system of coordinates Laplace's equation is

$$\nabla^2 c \equiv \left\{ \frac{\partial}{\partial r}\left[(r^2 - f^2)\frac{\partial c}{\partial r}\right] + \frac{1}{\sin\theta}\frac{\partial}{\partial\theta}\left[\sin\theta\frac{\partial c}{\partial\theta}\right] + \left[\frac{f^2}{r^2 - f^2} + \frac{1}{\sin^2\theta}\right]\frac{\partial^2 c}{\partial r^2}\right\} / (r^2 - f^2\cos^2\theta) = 0. \tag{3}$$

Using the method of separation of variables, one obtains two general solutions in the form of infinite series, one for the region interior to the ellipsoid and one converging in the external region. In this order, they are

$$c_i = \sum_{n=0}^{\infty}\sum_{m=0}^{n} P_n^m\left(\frac{r}{f}\right) T_n^m(\cos\theta)(A_n^m \cos m\phi + B_n^m \sin m\phi) \tag{4}$$

$$c_e = \sum_{n=0}^{\infty}\sum_{m=0}^{n} Q_n^m\left(\frac{r}{f}\right) T_n^m(\cos\theta)(C_n^m \cos m\phi + D_n^m \sin m\phi) \tag{5}$$

where P_n^m, Q_n^m are the associated Legendre functions of the first and second kind, and the T_n^m are the Ferrers associated functions, while the A, B, C, and D are arbitrary constants.

In these coordinates the normal derivative at an ellipsoidal surface S (given by $r=a$) depends simply on the derivative with respect to r;

$$\frac{\partial}{\partial n} = \frac{b}{(a^2 - f^2\cos^2\theta)^{1/2}}\left.\frac{\partial}{\partial r}\right|_{r=a} \tag{6}$$

where, as hereafter, $b=(a^2-f^2)^{1/2}$, the semi-minor axis of S.

Now consider the function, defined on S,

$$W = -(a^2 - f^2\cos^2\theta)^{1/2}\frac{\partial c_1}{\partial n}. \tag{7}$$

For a wide class of functions (including all continuous ones) this may be expanded as

$$W = \sum_{n=0}^{\infty}\sum_{m=0}^{n} T_n^m(\cos\theta)(E_n^m \cos m\phi + F_n^m \sin m\phi). \tag{8}$$

It is then seen that conditions (1) are satisfied by c_2 of the form (5) with

$$C_n^m = E_n^m \frac{f}{b}\left[dQ_n^m\left(\frac{a}{f}\right)/d\left(\frac{a}{f}\right)\right]$$
$$D_n^m = F_n^m \frac{f}{b}\left[dQ_n^m\left(\frac{a}{f}\right)/d\left(\frac{a}{f}\right)\right]. \tag{9}$$

If an expansion for the original field of the form

$$c_1 = \sum_{n=0}^{\infty}\sum_{m=0}^{n} T_n^m(\cos\theta)[J_n^m(r)\cos m\phi + K_n^m(r)\sin m\phi] \tag{10}$$

is available, then

$$E_n^m = -bJ_n^{m'}\Big|_{r=a}$$
$$F_n^m = -bK_n^{m'}\Big|_{r=a} \tag{11}$$

and if c_1 is harmonic so that it can be expanded in the form (4), then

$$C_n^m = -\left[P_n^{m'}\left(\frac{a}{f}\right)/Q_n^{m'}\left(\frac{a}{f}\right)\right]A_n^m$$
$$D_n^m = -\left[P_n^{m'}\left(\frac{a}{f}\right)/Q_n^{m'}\left(\frac{a}{f}\right)\right]B_n^m. \tag{12}$$

The primes denote derivatives with respect to the arguments.

In the case of (10) we may add c_1 and c_2 to get the expansion for c on the surface S of the form used in (8), with a typical term as

$$X_n^m \cos n\phi + Y_n^m \sin m\phi \tag{13}$$

where

$$X_n^m = J_n^m(a) - fJ_n^{m'}(a)\frac{Q_n^m}{Q_n^{m'}}. \tag{14}$$

Further, in the harmonic case, (12), we have

$$X_n^m = A_n^m[P_n^m - Q_n^m P_n^{m'}/Q_n^{m'}]$$

which, because of the relation (Hobson, '31)

$$P_n^m Q_n^{m'} - P_n^{m'} Q_n^m = (\text{constant})\cdot\frac{f^2}{b^2} = -\beta(n,m)\frac{f^2}{b^2}, \tag{15}$$

simplifies to

$$X_n^m = -f^2\beta(n,m)A_n^m/Q_n^{m'}\cdot b^2 \tag{16}$$

and similarly for Y_n^m. Here the omitted arguments are all a/f.

II: FORCES AND TORQUES

The concentration, c, varies over the surface of the ellipsoid, and a normal surface pressure depending on the concentration will produce a resultant force and torque on the ellipsoid. For a pressure of the form

$$p = kc \tag{17}$$

which (with $k = RT/M$; M = molecular wt. of the dissolved substance) is the usual diffusion-pressure-concentration relation for dilute solutions, it will be shown that the torque and force components involve only a few of the coefficients X, Y in the expansion of c in the form (8), (13). If the pressure-concentration relation is more complicated, (17) may be regarded as one term from the power series expansion of $p(c)$.

For the i-th component of the force on unit area of surface we have

$$f_i = -p \cos(nx_i), \tag{18}$$

and for the i-th component of the torque about the ellipsoid center contributed by the force on unit area

$$m_{jk} = x_j f_k - x_k f_j. \tag{19}$$

The total force and central torque are given by integrals over the ellipsoid surface

$$\begin{aligned} F_i &= \iint f_i dS \\ M_{jk} &= \iint m_{jk} dS. \end{aligned} \tag{20}$$

The component $M_{xy} = 0$, since at each point the pressure acts along a line intersecting the z-axis. Neglecting the coefficients, the expressions under the integral signs are respectively of the form

$$\sum_{m=0}^{\infty} \sum_{0}^{n} T_n^m(\cos\theta) \cos m\phi \cos(nx_i) dS \tag{21}$$

$$\sum_{m=0}^{\infty} \sum_{0}^{n} T_n^m(\cos\theta) \cos m\phi [x_j \cos(nx_k) - x_k \cos(nx_j)] dS \tag{22}$$

and similar expressions in $\sin(m\phi)$; while it may be verified that

$$\begin{aligned} \cos(nx) dS &= ab \sin^2\theta \cos\phi \, d\theta d\phi \\ \cos(ny) dS &= ab \sin^2\theta \sin\phi \, d\theta d\phi \\ \cos(nz) dS &= b^2 \sin\theta \cos\phi \, d\theta d\phi. \end{aligned} \tag{23}$$

The integration is from 0 to π for θ and 0 to 2π for ϕ. Upon integrating with respect to ϕ most of the terms vanish because of the orthogonality of the trigonometric functions. Surviving are F_x and F_y terms for $m = 1$, F_z for $m = 0$,

M_{zz} and M_{yz} for $m=1$; the integration yielding factors π, 2π, and π respectively. It should be noted at this point that an additive constant in the pressure will give no contribution to the forces or torque; integration over ϕ will leave its contribution to F_z only, and this will vanish on integrating with respect to θ, since the integrand will be $\sin\theta\cos\theta$, and the limits 0 and π.

The integration over θ proceeds in similar fashion, with somewhat more complicated treatment; but again most terms vanish, this time by the orthogonality of the Legendre functions. The surviving integrands are of the form

$$\begin{aligned} &\sum_{n=0}^{\infty} T_n^0(\cos\theta)\sin\theta\cos\theta\,d\theta \\ &\sum_{n=0}^{\infty} T_n^2(\cos\theta)\sin^2\theta\,d\theta \\ &\sum_{n=0}^{\infty} T_n^2(\cos\theta)\sin^2\theta\cos\theta\,d\theta. \end{aligned} \tag{24}$$

We now transform these by means of the definitions of the Ferrers harmonics and Legendre functions, together with two of the customary recursion formulae (MacRobert, '27):

$$\begin{aligned} P_1'(u) &= P_0(u) \\ P_2'(u) &= 3P_1(u) \end{aligned} \tag{25}$$

with $u=\cos\theta$, the prime ($'$) denoting differentiation with respect to u. Then formulae (24) become:

$$\begin{aligned} &-\sum_{n=0}^{\infty} P_n(u)P_1(u)du \\ &-\sum_{n=0}^{\infty} T_n^1(u)T_1^1(u)du \\ &-\sum_{n=0}^{\infty} T_n^1(u)T_2^1(u)du/3. \end{aligned} \tag{26}$$

The integration with respect to u is from -1 to 1. We use the orthogonality relations (MacRobert, '27):

$$\begin{aligned} &\int_{-1}^{1} P_n(u)P_r(u)du = 0\ (r\neq n);\quad 2/(2n+1)\ (r=n) \\ &\int_{-1}^{1} T_n^1(u)T_r^1(u)du = 0\ (r\neq n);\ 2n(n+1)/(2n+1)\ (r=n) \end{aligned} \tag{27}$$

and the surviving terms in (26) are for $n=1$, $n=1$ and $n=2$ respectively, integration giving factors 2/3, 4/3, and 4/5.

Finally, collecting all factors, we obtain the expressions which we have been seeking

$$
\begin{aligned}
F_x &= -4\pi abkX_1^1/3 \\
F_y &= -4\pi abkY_1^1/3 \\
F_z &= -4\pi b^2kX_1^0/3 \\
M_{zx} &= -4\pi bf^2kX_2^1/5 \\
M_{yz} &= 4\pi bf^2kY_2^1/5 \\
M_{xy} &= 0.
\end{aligned} \tag{28}
$$

III: SOME SPECIAL FIELDS

We consider now three particular c_1 fields of interest.

Constant gradient field.—One of the simplest cases is a field which has a constant gradient, and is hence Laplacian. Without loss of generality we may take

$$c_1 = gz + hx = gr\cos\theta + h(r^2 - f^2)^{1/2}\sin\theta\cos\phi \tag{29}$$

where g and h are constants. But $\cos\theta = T_1^0(\cos\theta)$; $\sin\theta = T_1^1(\cos\theta)$; $r = fP_1^0(r/f)$; and $(r^2-f^2)^{1/2} = fP_1^1(r/f)$. We have therefore immediately for the non-zero coefficients in the expansion (4) of this field

$$
\begin{aligned}
A_1^0 &= gf \\
A_1^1 &= hf.
\end{aligned} \tag{30}
$$

Now* $\beta(1,0) = 1$, $\beta(1,1) = -2$. Hence, from (16), (28) and (30),

$$
\begin{aligned}
F_x &= -8\pi af^3kh/3bQ_1^{1\prime} \\
F_z &= 4\pi f^3kg/3Q_1^{0\prime} \\
F_y &= 0 \\
M_{zx} &= 0 \\
M_{yz} &= 0 \\
M_{xy} &= 0.
\end{aligned} \tag{31}
$$

The torque about the center is identically zero, but not about other points. For example at the point $(0, 0, -a)$ there is a torque component $M_{xx}^* = aF_z$. It should be noted that the resultant force on the ellipsoid does not, in general, have the same direction as the original gradient. Only when the major axis is parallel to, or perpendicular to, the gradient, is the force along the same line (for $k>0$ in (17), the force is then in the opposite sense from the gradient).

Parabolic field.—We consider next a field of the form

$$c_1 = AR^2 \tag{32}$$

where R is the distance from a fixed point P. Without loss of generality the coordinate system may be taken so that the coordinates of P are $(x_0, 0, z_0)$. Expression (32) may be readily written in the form (10) by introducing (2) into

$$R^2 = (x - x_0)^2 + y^2 + (z - z_0)^2$$

and proceeding somewhat as was done in the previous case, (29). The resulting non-zero coefficients are

$$
\begin{aligned}
J_0^0 &= r^2 + x_0^2 + z_0^2 - 2f^2/3 \\
J_1^0 &= -2rz_0 \\
J_2^0 &= 2f^2/3 \\
J_1^1 &= -2x_0(r^2 - f^2)^{1/2};
\end{aligned} \tag{33}
$$

hence, by (14) and (28), F_y and all the central torque components are identically zero.

Point source field.—The symmetric field of a point source

$$c_1 = B/R \tag{34}$$

where R is the distance from the source point P to the field point, is harmonic and (for P outside the ellipsoid) may be expanded in the form (4). Without loss of generality we may choose axes (passing the xy-plane through P) so that the coefficients are, for $m \geqq 1$,

$$A_n^m = 2(2n+1)(-1)^m B[(n-m)!/(n+m)!]^2 Q_n^m\left(\frac{r_1}{f}\right) T_n^m(\cos\theta_1)/f; \tag{35}$$

with A_n^0 one half of expression (35) for $m=0$. Herein $(r_1;\ \theta_1;\ 0)$ are the ellipsoidal coordinates of P. For these, and the general formulae with $\phi_1 \neq 0$, see reference to MacRobert, '27. In (15), $\beta(2, 1) = -6$. Using (16), (28), and (35), we obtain

$$
\begin{aligned}
F_x &= \gamma H \sin\theta_1 \\
F_y &= 0 \\
F_z &= \gamma L \cos\theta_1 \\
M_{zx} &= \gamma K \sin\theta_1 \cos\theta_1 \\
M_{xy} &= 0 \\
M_{yz} &= 0,
\end{aligned} \tag{36}
$$

where

$$
\begin{aligned}
\gamma &= 4\pi kf/b \\
H &= aBQ_1^1(r_1/f)/Q_1^{1\prime} \\
L &= bBQ_1^0(r_1/f)/Q_1^{0\prime} \\
K &= f^2BQ_2^1(r_1/f)/Q_2^{1\prime}.
\end{aligned} \tag{37}
$$

The omitted arguments are here, as throughout, a/f.

If we think of the ellipsoid as pinned at the center, but free to rotate, there will be certain equilibrium orientations. The condition for these is

$$M_{zz} = 0 \tag{38}$$

the other torque components being identically zero. By (36), this is

$$\sin \theta_1 \cos \theta_1 = 0 \tag{39}$$

which is satisfied, in the range $0 \leqq \theta \leqq \pi$, by

$$\theta_1 = 0 \text{ or } \pi \tag{40}$$

$$\theta_1 = \pi/2. \tag{41}$$

For the equilibrium to be stable, there is the additional requirement that the torque on the ellipsoid near the equilibrium position be in such direction as to cause return to that position; i.e.,

$$dM_{zz}/d\theta_1 > 0. \tag{42}$$

In the present case, supposing $k>0$, this is

$$K(\cos^2 \theta_1 - \sin^2 \theta_1) > 0; \tag{43}$$

hence for the equilibrium position described by (40) to be stable we must have $K>0$; while for that of (41), $K<0$. Thus in such a situation the ellipsoid will align itself with its long axis along the line to the source point P if $K>0$, and perpendicular to that line if $K<0$.

If the ellipsoid is pinned at the point $(0, 0, -pa)$, the moment is

$$M_{zz}^* = M_{zz} + paF_z: \tag{44}$$

and, corresponding to (39), equilibrium requires

$$K \sin \theta_1 \cos \theta_1 + paH \sin \theta_1 = 0 \tag{45}$$

with solutions

$$\theta_1 = 0 \text{ or } \pi \tag{46}$$

and, if $|paH/K| \leqq 1$,

$$\theta_1 = \arccos(-paH/K). \tag{47}$$

IV: NATURE OF THE Q FUNCTIONS, AND SOME CONSEQUENCES

The Q functions appearing above are not very familiar, and a few remarks concerning their behavior may be desirable. All $Q_n^m(x)$ become infinite at $x=1$ and vanish at $x=\infty$. A scheme for actual computation of those here needed may be considered as given by the relations (MacRobert, '27)

$$\begin{aligned} Q_0(x) &= 1/2 \log [(x+1)/(x-1)] \\ Q_1 &= Q_1^0 = xQ_0 - 1 \\ Q_2 &= (3xQ_1 - Q_0)/2 \\ Q_n^1 &= n(x^2-1)^{-1/2}(xQ_n - Q_{n-1}) \\ \frac{d}{dx} Q_n^1 &= n(x^2-1)^{-3/2}[(nx^2 - n - 1)Q_n - xQ_{n-1}]. \end{aligned} \tag{48}$$

* See Section IV of this paper.

By carrying through such a procedure on the series expression

$$Q_0 = \sum_0^\infty 1/(2n+1)x^{2n+1}, \tag{49}$$

it may be shown that as x increases from 1 to ∞, Q_1^0 decreases monotonically from ∞ to 0, while Q_2^1 and Q_1^1 increase monotonically from $-\infty$ to 0. Their derivatives are thus of opposite sign to the functions themselves; and it follows that H, K, and L of (37) are opposite in sign to B. The signs in (31) are similarly found, and it is seen that the force components are in opposite direction from the components of the original gradient.

For large x,

$$\begin{aligned} Q_1^0 &= 1/3x^2 \\ Q_1^1 &= -2/3x^2 \\ Q_2^1 &= -2/5x^3 \end{aligned} \tag{50}$$

approximately. Using (50) and the corresponding first terms, x, x, and $3x^2$, of P_1^0, P_1^1, and P_2^1, one can evaluate $\beta(n, m)$ at $x=\infty$; the results being as given in section number III. Note that these values are for the range $1<x<\infty$; there are changes in sign across the singular point $x=1$

It can be shown that

$$\alpha(x) = Q_1^{1\prime}/Q_2^{1\prime}$$

has the limit unity at $x=1$.

Now consider the ratio of the two torque terms in (44). For a case where the point source is distant from the ellipsoid, so that $r_1 \gg f$, we have, using (50),

$$\phi \equiv K/paH = 3f^3\alpha(a/f)/5a^2pr_1. \tag{51}$$

As f varies from 0 to a, the ellipsoid shrinks from a sphere into a line segment; while $x=a/f$ ranges from ∞ to 1. At $x=1$, $\phi=3a/5pr_1$, which, since $r_1 \gg a$, is negligible except when p is very small. At $x=\infty$, $\phi=2f^2/3apr_1$ and so vanishes for a sphere as it should be symmetry. For intermediate eccentricities computation again shows that ϕ is negligible except for p very small. It is thus seen that unless the ellipsoid be pinned very near the center, the central torque arising from a distant point source is far outweighed by the moment of the force, and only radial orientation, (46), is possible. The corresponding formulae for an oblate ellipsoid of revolution are obtained by replacing f by f/i. For imaginary arguments, Q_1^1, Q_1^0, and $Q_2^{1\prime}$ are real; while $Q_1^{1\prime}$, $Q_1^{0\prime}$, and Q_2^1 are imaginary. It is thus seen that expressions (31) and (37) all remain real.

V: APPLICATIONS

If the original field is determined by a Poisson equation,

$$\nabla^2 c_1 = \rho(x, y, z), \tag{52}$$

together with boundary conditions at infinity, then the image field c_2 specified by (1) is such that the resulting field $c=c_1+c_2$ satisfies the same Poisson equa-

tion,* the same conditions at infinity, and the additional boundary conditions on S.

Now a steady state diffusion in a region of constant diffusion coefficient D is governed by the equation

$$D\nabla^2 c = q$$

where q is the consumption rate. In general there will be several substances c_i with coupled reactions, so that $q = q\ (c_1 \cdots c_n)$ and there is an entire system of coupled and (since q is of the nature of a product of powers of the concentrations of the substances involved) non-linear equations;

$$D_i \nabla^2 c_i = q_i. \tag{53}$$

Not much progress has been made with such problems. However, the substitution of space functions for the q_i has been used with some success by N. Rashevsky ('36a), and H. Landahl ('37), even in such coupled systems; and is a useful procedure in successive approximation schemes. This gives the form (52), which is thus of interest in such problems.

These remarks will serve to indicate the significance of the conditions (1): a) and b) suppose that $\rho = q/D$ outside S is unaffected by the image field, and that S is at a great distance from other surfaces with boundary conditions; while c) states that S is inactive, i.e., that there is no diffusion to or from it. If the surface S is near other particles or surfaces the problem is more difficult, but the present formulation may sometimes be used as a single step in a successive approximation solution, as has been done, for example, in the case of the two spheres or two parallel cylinders (Young, '36). If there are surface reactions on S, c) would be modified accordingly. The situation is still more complex for volume reaction cells bounded by S.

The specific fields studied above are of interest as follows:

Constant gradient field.—This may be useful as a simple approximation in any case where an ellipsoid is pretty well isolated in a c_1 field which does not vary rapidly in the neighborhood of the ellipsoid. It is to be noted, however, that while the approximation may be serviceable as regards the resultant force, it is too rough to give an estimate for the central torque.

Parabolic field.—This is the form of the field in the interior of a simple constant-consumption spherical cell (Rashevsky, '36b), and, while the presence of the cell wall at a finite distance makes the above results only approximate, they may have application in the theory of cellular structure and behavior. To the just mentioned approximation, an ellipsoidal particle immersed in such a cell will experience no torque about its center.

Point source field.—This is the type of field outside a spherical cell (Rashevsky, '36b), and likewise in the membrane if there is no consumption therein. This suggests application to the particles comprising the membrane itself; i.e., to the theory of membrane structure, permeability, etc.

For a substance consumed by the cell, B of (34) is negative; for a produced substance it is positive. A centrally pinned prolate ellipsoid would thus tend

* Outside the ellipsoid S.

to alignment along the radius outside a consuming cell, perpendicular to the radius outside a producing one. However for values appropriate to cellular and colloidal systems (say $r_1 = 10^{-3}$ cm., $b = 10^{-7}$ cm.), ϕ of (51) is negligible and the transverse orientation disappears for any but the most exact central pinning.

Combination field.—In the more general case of a spherical cell with several concentric regions, in each of which the reaction rate and diffusion coefficient are constant, the variable field in each region is of the form

$$AR^2 + B/R. \tag{54}$$

For the innermost region $B = 0$, for the others it in general is not. Thus ellipsoids pinned exactly at their centers would be expected to be oriented in all but the central region. If B has different sign in the different regions, this orientation will differ accordingly; being radial where $B < 0$, and transverse where $B > 0$.

VI: CONCLUDING REMARKS

It should be noted that, insofar as equations of type (52) apply, each of several diffusing substances may be considered separately, and their torques and forces simply added for each particle. The above discussion of pinned ellipsoids represents a particular simple case introduced for illustrative purposes to show the tendency of these forces acting alone; it does not apply in general. In applications, any forces and torques of the type here discussed are to be added to any others which may be present (e.g. electrical and other constitutive forces in structures such as membranes; electrical forces on colloidal particles; surface tension effects; etc.), and it is in terms of the total resultant that equilibrium orientation, etc., must be discussed.

Provided that there is actually a pressure effective mechanically in the way described by (17), there will, in general, be forces tending to move a free particle. The same, with the further complication introduced by their several boundaries, is true for a number of particles; and this may find application to such phenomena as protoplasmic streaming, granular movement, and the like. The case of actual motion, is, however, complicated by convection currents, hydrodynamic resistance, etc., and will not be discussed here.

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MATHEMATICAL BIOPHYSICS OF CELL RESPIRATION

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I

In the articles entitled—Studies on the Mathematical Biophysics of Metabolizing Systems with Reference to Living Cells (Rashevsky, '35a, b, '36), a formula was developed for the consumption of oxygen at various oxygen concentrations. Those studies form one particular aspect of the more general problems, which arise in connection with the physico-mathematical studies of cellular growth and multiplication. In the following study, the treatment of the oxygen consumption by simple cells is revised and extended. Experiment and theory are discussed in connection with two sets of data.

We consider here an idealized, abstract picture of a cell, which has spherical symmetry and a constant diffusion coefficient D_i and permeability h_i for each substance. Regarding metabolism, the following assumptions are made:* (1) The glucose breaks down at a rate proportional to its own concentration, c_1, forming lactic acid, which (2) resynthesizes to form glucose at a rate proportional to the square of its own concentration c_2, (3) the lactic acid is oxidized at a rate proportional to the product of its own concentration with that of oxygen c_3.

$$C_6H_{12}O_6 \underset{bc_2^2}{\overset{ac_1}{\rightleftarrows}} 2C_3H_6O_3$$

$$C_3H_6O_3 + 3O_2 \xrightarrow[fc_2c_3]{} 3CO_2 + 3H_2O.$$

The break-down of the glucose is considered as spontaneous whereas the resynthesis and oxidation processes are considered reactions due to molecular collision. Any intermediate steps are not taken into account since the products would not appreciably leave the cell.

Letting the concentrations c_i be given in grams per cubic centimeter and noting that the ratio of the molecular weight of lactic acid to that of oxygen, $(3O_2)$, is $n \equiv .94$, we may write the equations as follows:

(1) Glucose: *Consumed* proportional to its own concentration, ac_1.
Produced proportional to the square of the concentration of lactic acid, bc_2^2.

(2) Lactic Acid: *Consumed* proportional to the square of its own concentration, bc_2^2.
Consumed proportional to the product of its own concentration with that of oxygen, nfc_2c_3.
Produced proportional to the concentration of glucose, ac_1.

* The same assumption regarding the cell and its metabolism were made by N. Rashevsky, except that the Pasteur-Meyerhof reaction is included here.

(3) Oxygen: *Consumed* proportional to the product of its own concentration with that of oxygen, fc_2c_3.

For the stationary state, the differential equations and boundary conditions then become:

$$D_1 \nabla^2 c_1 = ac_1 - bc_2^2 \tag{1}$$

$$D_2 \nabla^2 c_2 = nfc_2c_3 - ac_1 + bc_2^2 \tag{2}$$

$$D_3 \nabla^2 c_3 = fc_2c_3 \tag{3}$$

$$D_i' \nabla^2 c_i' = 0 \tag{4}$$

$$D_i \frac{dc_i}{dr} = h_i(c_i' - c_i) = D_i' \frac{dc_i'}{dr} \quad \text{at} \quad r = r_0 \tag{5}$$

$$\nabla^2 c \equiv \frac{1}{r} \frac{d^2(cr)}{dr^2} \tag{6}$$

where the prime indicates the value outside of the cell, that is, for $r > r_0$. The equations for CO_2 and H_2O which are a part of the system do not concern us here and so are omitted (Rashevsky, '35b).

In approximating a solution we shall follow the method used by N. Rashevsky ('36).

The solution of equation $D\nabla^2 C = ac$ within the cell, assuming spherical symmetry, is:

$$c = \frac{A}{r} \sinh r\sqrt{\frac{a}{D}} = A\sqrt{\frac{a}{D}}\left(1 + \frac{ar^2}{6D} + \frac{3a^2r^4}{40D^2} + \cdots\right).$$

By neglecting the fourth and higher powers of r in the expansion, we have a solution which would have resulted had we assumed a constant consumption of the substance of a certain average value q. The value of q would then be given by the volume average of $ac(r)$, or

$$q = \frac{1}{V}\int_0^V acdV = \frac{3a}{r_0^3}\int_0^{r_0} cr^2 dr \tag{7}$$

We are then fitting a parabola to each concentration in terms of the boundary conditions. Since experimentally we would take the parabola of the best average fit, the constants derived would not have the accuracy implied by the comparison of the experimental to the theoretical curve. The concentration at the surface might be many times larger than at the center and still be fairly well approximated by a parabola. The consumption curve would then fit well with the theoretical curve while the constants derived would only be valid as orders of magnitudes.

A measure of the upper limit of the error due to the approximation may be obtained. It would be given by the difference between the rate of consumption and that rate for which r_0 is put equal to zero. This gives the deviation of the extreme values from the average. The average error, under normal conditions,

is then of the order of five per cent in some cases and much less in others. At low values of oxygen tension and the external concentration of lactic acid, however, this estimate may give a hundred per cent error while the agreement between theory and experiment is within a few per cent.

Following this proceedure of fitting a parabola to each concentration, we have for our equations

$$D_i \nabla^2 c_i = q_i \qquad (i = 1, 2, 3) \tag{8}$$

$$D_i' \nabla^2 c_i' = 0 \tag{9}$$

which with the boundary conditions, equations (5), give the solution for the concentrations as functions of position and in terms of the constants of the cell.

$$c_i = A_i + B_i r^2 \tag{10}$$

$$c_i' = c_{0i} - \frac{q_i r_0^3}{3D_i'} \frac{1}{r} \tag{11}$$

where

$$A_i = c_{0i} - \left(\frac{r_0^2}{6D_i} + \frac{r_0^2}{3D_i'} + \frac{r_0}{3h_i}\right) q_i = c_{0i} - m_i q_i \tag{12}$$

$$B_i = \frac{q_i}{6D_i}. \tag{13}$$

As in equation (7) we obtain the average rates of consumption (Rashevsky, '36).

$$q_1 = \frac{3}{r_0^3} \int_0^{r_0} (ac_1 - bc_2^2) r^2 dr \tag{14}$$

$$q_2 = \frac{3}{r_0^3} \int_0^{r_0} (nfc_2c_3 - ac_1 + bc_2^2) r^2 ar \tag{15}$$

$$q_3 = \frac{3}{r_0^3} \int_0^{r_0} fc_2c_3 r^2 dr. \tag{16}$$

Expanding and integrating equation (14)

$$\begin{aligned} q_1 &= \frac{3}{r_0^3} \int_0^{r_0} [aA_1 + aB_1 r^2 - bA_2^2 - 2bA_2B_2 r^2 - 6B_2^2 r^4] r^2 dr \\ &= aA_1 + \frac{3}{5} aB_1 r_0^2 - bA_2^2 - \frac{6}{5} bA_2B_2 r_0^2 - \frac{3}{7} bB_2^2 br_0^4. \end{aligned} \tag{17}$$

Substituting (12) and (13) in (17) we have

$$q_1 = ac_{01} - bc_{02}^2 - am_1q_1 + \frac{ar_0^2}{10D_1}q_1 + 2bc_{02}m_2q_2$$
$$- \frac{br_0^2}{5D_2}c_{02}q_2 + \frac{br_0^2}{5D_2}m_2q_2^2 - bm_2^2q_2^2 - \frac{br_0^4}{84D_2^2}q_2^2. \tag{18}$$

Now equation (15) can be written

$$q_2 = nq_3 - q_1. \tag{19}$$

Expanding and integrating equation (16)

$$q_3 = \frac{3f}{r_0^3}\int_0^{r_0}[A_2A_3 + A_2B_3r^2 + A_3B_2r^2 + B_2B_3r^4]r^2dr$$
$$= fA_2A_3 + \frac{3}{5}f(A_2B_3 + A_3B_2)r_0^2 + \frac{3}{7}fB_2B_3r_0^4. \tag{20}$$

Substituting (12) and (13) in the above, we have

$$q_3 = fc_{02}c_{03} - fc_{02}m_3q_3 - fc_{03}m_2q_2 + \frac{fr_0^2}{10D_2}c_{03}q_2$$
$$+ \frac{fr_0^2}{10D_3}c_{02}q_3 + fm_2m_3q_2q_3 - \frac{fr_0^2}{10D_3}m_2q_2q_3$$
$$- \frac{fr_0^2}{10D_2}m_3q_2q_3 + \frac{fr_0^4}{84D_2D_3}q_2q_3. \tag{21}$$

Equations (18), (19) and (21) may now be written in the following form

$$Aq_2^2 - Bq_2 + Cq_1 - D = 0 \tag{22}$$

$$q_2 + q_1 - nq_3 = 0 \tag{23}$$

$$Hq_2q_3 - Kq_3 - Mq_2 + S = 0. \tag{24}$$

Putting $\Lambda_i = \frac{r_0^2}{15D_i} + \frac{r_0^2}{3D_i'} + \frac{r_0}{3h_i}$ then

$$A = b\Lambda_2^2 + \frac{br_0^4}{525D_2^2} = b\Lambda_2^2(1 + \epsilon_1)$$
$$B = 2bc_{02}\Lambda_2$$
$$C = a\Lambda_1 + 1$$
$$D = ac_{01} - bc_{02}^2$$
$$H = f\Lambda_2\Lambda_3 + \frac{fr_0^4}{525D_2D_3} = f\Lambda_2\Lambda_3(1 + \epsilon_2)$$
$$K = fc_{02}\Lambda_3 + 1 \tag{25}$$

$$M = fc_{03}\Lambda_2 = M_0 c_{03}$$
$$S = fc_{02}c_{03} = S_0 c_{03}.$$

Also $\epsilon_1 < 3/7$; $\epsilon_2 < 3/7$. As defined above

$$\epsilon_1 = \frac{r_0^4}{525 D_2^2 \Lambda_2^2} = \frac{r_0^2}{525 D_2^2 \left(\frac{r_0}{15D_2} + \frac{r_0}{3D_2'} + \frac{1}{3h}\right)^2}$$
$$= \frac{225}{525\left(1 + \frac{15D_2}{3D_2'} + \frac{15D_2}{3h_2 r_0}\right)^2}.$$

The value of ϵ_1 would be a maximum for indefinitely large values of h_2 and D_2' or for D_2 equal to zero. Hence $\epsilon_{1\max} = 3/7$. In the same way we arrived at the maximum value of ϵ_2.

Solving for q_1 in (23) and substituting in (22)

$$Aq_2^2 - (B + C)q_2 + nCq_3 - D = 0. \tag{26}$$

Solving for q_2 in (24) and substituting in the above, we obtain

$$A\left(\frac{Kq_3 - S}{Hq_3 - M}\right)^2 - (B + C)\left(\frac{Kq_3 - S}{Hq_3 - M}\right) + nCq_3 - D = 0 \tag{27}$$

or clearing of fractions

$$\begin{aligned} &nCH^2 q_3^3 + [AK^2 - (B + C)HK - DH^2 - 2nCHM]q_3^2 \\ &\quad + [(B + C)(HS + KM) - 2AKS + 2DHM + nCM^2]q_3 \\ &\quad + [AS^2 - (B + C)SM - DM^2] = 0. \end{aligned} \tag{28}$$

Now, for large values of c_{03}, i.e., large M and S, equation (28) becomes

$$nCM^2 q_{3l} + AS^2 - (B + C)SM - DM^2 = 0$$

or the limiting value of the oxygen consumption for large oxygen pressures is given by

$$\begin{aligned} q_{3l} &= \frac{-AS_0^2 + (B + C)S_0 M_0 + DM_0^2}{nCM_0^2} \\ &= \frac{1}{n(a\Lambda_1 + 1)\Lambda_2}\left[(a\Lambda_1 + 1)c_{02} + ac_{01}\Lambda_2 - bc_{02}^2\Lambda_2\epsilon_1\right]. \end{aligned} \tag{29}$$

The existence of a limiting rate of consumption is not unexpected. For values of c_3, small compared with c_2, an approximately linear relation between c_3 and the oxygen consumption would be expected since the small consumption would only slightly affect c_2. But with increasingly large values of c_3, the consumption would increase to a value at which the lactic acid would be oxidized as rapidly

as it is formed by the break-down of glucose. Increase in the oxygen tension would then not raise the consumption beyond this value.

Now let $y = q_3/q_{3l}$ and $c_{03} = x$ Substituting in equation (28), we obtain

$$\begin{aligned} &nCH^2q_{3l}^2y^3 + [AK^2 - (B + C)HK - DH^2 - 2nCHM_0x]q_{3l}y^2 \\ &+ x[(B + C)(HS_0 + KM_0) - 2AKS_0 + 2DHM_0 + nCM_0^2x]y \\ &- nCM_0^2x^2 = 0. \end{aligned} \tag{30}$$

Rearranging in terms of x

$$\begin{aligned} &nCM_0^2(1 - y)x^2 - [(B + C)(HS_0 + KM_0) - 2AKS_0 + 2DHM_0]xy \\ &+ 2nCHM_0q_{3l}xy^2 + [-AK^2 + (B + C)HK + DH^2]q_{3l}y^2 \\ &- nCH^2q_{3l}^2y^3 = 0. \end{aligned} \tag{31}$$

Expanding the coefficient of xy and adding and subtracting

$$\frac{2AHS_0^2}{M_0}, \qquad BHS_0,$$

and BKM_0 we obtain it in the form

$$\frac{1}{M_0}(KM_0 - HS_0)[(B + C)M_0 - 2AS_0] + 2\frac{H}{M_0}q_{3l}nCM_0^2.$$

Now letting

$$\alpha = \frac{(KM_0 - HS_0)[(B + C)M_0 - 2AS_0]}{nCM_0^3} \tag{32}$$

$$\beta = \frac{H}{M_0}q_{3l} \tag{33}$$

$$\gamma = \frac{(B + C)HK + DH^2 - AK^2}{nCHM_0} = \alpha + \beta - \delta \tag{34}$$

$$\delta = \frac{A}{nCHM_0^3}(HS_0 - KM_0)^2 \tag{35}$$

we may rewrite equation (31) in the following form

$$(1 - y)x^2 - (\alpha + 2\beta)xy + 2\beta xy^2 + \beta\gamma y^2 - \beta^2y^3 = 0. \tag{36}$$

Substituting equations (25) in (32), (33) and (35), they become

$$\alpha = \frac{1}{nf\Lambda_2}\left[1 - \frac{2bc_{02}\Lambda_2\epsilon_1}{(a\Lambda_1 + 1)}\right][1 - fc_{02}\Lambda_3\epsilon_2] \tag{37}$$

$$\begin{aligned} \beta &= \Lambda_3q_{3l}(1 + \epsilon_2) \\ \gamma &= \alpha + \beta - \delta \end{aligned} \tag{38}$$

$$\delta = \frac{b}{nf^2(a\Lambda_1 + 1)\Lambda_3} \frac{(1 + \epsilon_1)}{(1 + \epsilon_2)} (1 - fc_{02}\Lambda_3\epsilon_2)^2. \tag{39}$$

Solving for x and choosing the plus sign for the radical since for $y = 1$, $x = \infty$, we obtain

$$x = \frac{(\alpha + 2\beta)y - 2\beta y^2 + \sqrt{(\alpha y + 2\beta y - 2\beta y^2)^2 - 4(1 - y)(\beta\gamma y^2 - \beta^2 y^3)}}{2(1 - y)}$$

which with equation (34) reduces to

$$x = \beta y + \frac{\alpha y}{2(1 - y)}\left[1 + \sqrt{1 + \frac{4\beta\delta}{\alpha^2}(1 - y)}\right]. \tag{40}$$

For the data with which the theory is compared, the conditions may be more closely approximated by somewhat different and less general assumptions. The results are sufficiently similar so that application and discussion of the following ing may readily be applied to the preceding.

II

Let us now consider a system in which the glucose breaks down at a constant rate k, but does not appreciably change in quantity: This assumption may be justified for many experiments in which the cells were not kept in a sugar solution, but still survived the period without the external source of food. We may also set $D_i' = \infty$ since generally the suspensions of cells are kept under continuous motion.

Using the same proceedure as in section one, we have the differential equations and boundary conditions

$$D_2 \nabla^2 c_2 = nfc_2c_3 + bc_2^2 - k \tag{41}$$

$$D_3 \nabla^2 c_3 = fc_2c_3 \tag{42}$$

$$D_i' \nabla^2 c_i' = 0 \tag{43}$$

$$D_i \frac{dc_i}{dr} = h_i(c_{0i} - c_i) \tag{44}$$

and as before

$$D_i \nabla^2 c_i = q_i \tag{45}$$

$$D_i' \nabla^2 c_i' = 0 \tag{46}$$

for which the solutions are,

$$c_i = A_i + B_i r^2 \tag{47}$$

$$c_i' = c_{0i} \tag{48}$$

where

$$A_i = c_{0i} - \left(\frac{r_0^2}{6D_i} + \frac{r_0}{3h_i}\right)q_i = c_{0i} - m_i q_i \tag{49}$$

$$B_i = \frac{q_i}{6D_i}. \tag{50}$$

Following the procedure in section one, we have

$$\begin{aligned} q_2 &= \frac{3}{r_0^3}\int_0^{r_0}(nfc_2c_3 + bc_2^2 - k)r^2dr \\ &= nq_3 - k + bc_{02}^2 - 2bc_{02}m_2q_2 + \frac{br_0^2}{5D_2}c_{02}q_2 \\ &\quad + bm_2^2q_2^2 - \frac{br_0^2}{5D_2}m_2q_2^2 + \frac{br_0^4}{84D_2^2}q_2^2 \end{aligned} \tag{51}$$

and

$$\begin{aligned} q_3 &= \frac{3}{r_0^3}\int_0^{r_0} fc_2c_3r^2dr \\ &= fc_{02}c_{03} - fc_{02}m_3q_3 + \frac{fr_0^2}{10D_3}c_{02}q_3 - fc_{03}m_2q_2 \\ &\quad + \frac{fr_0^2}{10D_2}c_{03}q_2 + fm_2m_3q_2q_3 - \frac{fr_0^2}{10D_3}m_2q_2q_3 \\ &\quad - \frac{fr_0^2}{10D_2}m_3q_2q_3 + \frac{fr_0^4}{84D_2D_3}q_2q_3. \end{aligned} \tag{52}$$

Equations (51) and (52) may be written,

$$Aq_2^2 - (B+1)q_2 + nq_3 - D = 0 \tag{53}$$

$$Hq_2q_3 - Kq_3 - Mq_2 + S = 0. \tag{54}$$

Putting

$$\Lambda_i = \frac{r_0^2}{15D_i} + \frac{r_0}{3h_i}$$

then

$$\begin{aligned} A &= b\Lambda_2^2 + \frac{br_0^4}{525D_2^2} = b\Lambda_2^2(1+\epsilon_1) \\ B &= 2bc_{02}\Lambda_2 \\ D &= k - bc_{02}^2 \\ H &= f\Lambda_2\Lambda_3 + \frac{fr_0^4}{525D_2D_3} = f\Lambda_2\Lambda_3(1+\epsilon_2) \end{aligned} \tag{55}$$

$$K = 1 + fc_{02}\Lambda_3$$
$$M = fc_{03}\Lambda_2 = M_0c_{03}$$
$$S = fc_{02}c_{03} = S_0c_{03}.$$

Solving for q_2 in equation (54) and substituting in (53), then clearing and rearranging, we obtain

$$\begin{aligned} &nH^2q_3^3 + [AK^2 - (B+1)HK - H^2D - 2nHM]q_3^2 \\ &+ [(B+1)(KM + HS) - 2AKS + 2DHM + nM^2]q_3 \\ &+ [AS^2 - (B+1)MS - DM^2] = 0. \end{aligned} \tag{56}$$

This may be obtained directly from equation (28) section one, by replacing C by unity, as equation (26) then becomes equation (53).

Now let $y = q_3/q_{3l}$, and $c_{03} = x$. Equation (56) may then be written in the form

$$x^2(1-y) - (\alpha + 2\beta)xy + 2\beta xy^2 + \beta\gamma y^2 - \beta^2 y^3 = 0 \tag{57}$$

or solving for x

$$x = \beta y + \frac{\alpha y}{2(1-y)}\left[1 + \sqrt{1 + \frac{4\beta\delta}{\alpha^2}(1-y)}\right] \tag{58}$$

where

$$\alpha = \frac{1}{nf\Lambda_2}[1 - 2bc_{02}\Lambda_2\epsilon_1][1 - fc_{02}\Lambda_3\epsilon_2] \tag{59}$$

$$\beta = \Lambda_3(1+\epsilon_2)q_{3l} \tag{60}$$

$$\gamma = \alpha + \beta - \delta \tag{61}$$

$$\delta = \frac{b}{nf^2\Lambda_3}\frac{(1+\epsilon_1)}{(1+\epsilon_2)}(1 - fc_{02}\Lambda_3\epsilon_2)^2 \tag{62}$$

$$q_{3l} = \frac{1}{n\Lambda_2}[c_{02} + k\Lambda_2 - bc_{02}^2\Lambda_2\epsilon_1]. \tag{63}$$

These relations may be obtained from similar ones in section one by replacing $a\Lambda_1$ by zero and ac_{01} by k. This is evident when equations (25) and (55) are compared.

From the above equation (63), (see also equation (29)), we have a relation between the oxygen consumption and the external concentration of lactic acid, c_{03} being large. By comparing with an experimental relation between these two, the oxygen concentration being of constant, high, known value, we may obtain Λ_2, k and $b\epsilon_1$. The latter value gives a minimum value of b and may give its approximate value. Using this estimate of b, the equations (58), (59) and (60) may be revised if necessary, and new values of Λ_2 and k derived. A comparison of the two values of Λ_2 and k may serve as an indication of the validity of the approximations and assumptions made.

If the equation (29) is used and the additional relation between the oxygen

consumption and the concentration of glucose be known, we have a further check, and may determine the value of a and Λ_1.

For large values of y, we have on expanding (58) and using only the first approximation,

$$x = \frac{\alpha}{1-y} \tag{64}$$

and for small values of δ

$$x = \left(\beta + \frac{\beta\delta}{\alpha}\right) y + \frac{\alpha}{1-y}. \tag{65}$$

But for $\delta = 0$, equation (58) becomes

$$x = \beta y + \frac{\alpha y}{1-y}. \tag{66}$$

From equation (64) we see that the value of δ does not affect the shape of the curve for large values of y. Comparing equations (65) and (66), we see that for small values of δ, δ does not change the form of the curve, the only effect being that β is replaced by β'. For very large values of δ we have $x = y\sqrt{\beta\delta}/(1-y)$ the curve of which is somewhat similar in shape to the curve of equation (66). Hence the curve given by equation (58) can be closely approximated by equation (66) in which $\delta = 0$, and α and β are somewhat changed. Hereafter we shall take the case $\delta = 0$, where

$$x = \beta y + \frac{\alpha y}{1-y}$$

and

$$\left(\frac{dx}{dy}\right)_{y=0} = \alpha + \beta$$

$$x = \frac{\alpha}{1-y} \quad \text{for} \quad y \sim 1 \tag{67}$$

by which α and β may be evaluated from an empirical relation between x and y. Some conclusions may then be drawn concerning the magnitude of the coefficients of diffusion and permeability, and of the constants f and k.

III

Before discussing the application of the relations derived, it is convenient to make some further rough approximations and to rearrange some of the equations. Now we have put $b = 0$, $n \sim 1$, ϵ_1 and ϵ_2 cannot exceed three-sevenths and are probably much less. The values α, β, q_{3l} and r_0 are experimentally determined. Though the value of c_{02} is not given it is probably rather small—of the order of 10^{-4} to 10^{-6}, due to the diffusion of lactic acid from the cells. If this is

the case, the value of c_{02} would not have been constant throughout the experiment since it would be appreciable only at low oxygen tensions. However, this would not be expected to have a serious effect. Under these conditions equations (59), (60) and (63) may now be written approximately

$$f\Lambda_2 = \frac{1}{\alpha} \tag{68}$$

$$\Lambda_3 = \frac{\beta}{q_{3l}} \tag{69}$$

$$k = q_{3l}. \tag{70}$$

We also have

$$D_i \gtrsim \frac{r_0^2}{15\Lambda_i} \tag{71}$$

$$h_i \gtrsim \frac{r_0}{3\Lambda_i} \tag{72}$$

were one or both of the $\sim$ signs must hold. This is evident from the definition of Λ_i,

$$\Lambda_i = \frac{r_0^2}{15D_i} + \frac{r_0}{3h_i}$$

since one or both of the terms on the right must be of the order of Λ_i.

The order of magnitude of h_3 may also be obtained as follows. If Δc_3 is the difference in oxygen concentration across the membrane of the cell, we have for M, the amount entering the cell per minute,

$$M = \Delta c_3 h_3 4\pi r_0^2 = \frac{4}{3} q_{3l} \pi r_0^3 \tag{73}$$

or

$$\Delta c_3 = \frac{q_{3l} r_0}{3h_3}. \tag{74}$$

But Δc_3 cannot exceed the concentration c_3 at any point, i.e., $\Delta c_3 < c_{03}^*$, if $c_{03} = c_{03}^*$ where q_3 first approximates q_{3l}, being somewhat larger than $\alpha+\beta$ (see equation 67). Then

$$h_3 > \frac{q_{3l} r_0}{3c_{03}^*}. \tag{75}$$

IV

For each set of data, the relation with b set equal to zero was used. In each case of the Arbacia eggs (Harvey, '32, Amberson, '28, Tang and Gerard, '32,

Tang, '31) the agreement is well within the experimental error. But to get the agreement obtained for the bacteria (Shoup, '29) data it was necessary to

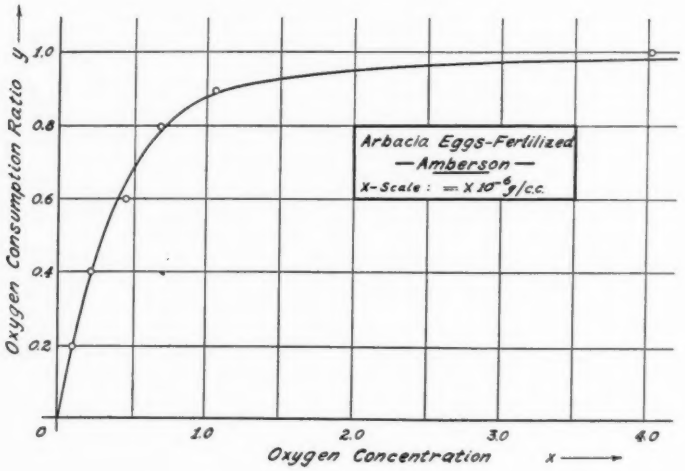

FIG. 1.

take the limiting value of the oxygen consumption two per cent higher than that given. This is equivalent to stating that the value of the oxygen consumption given by the curve for normal pressure (16 cm. Hg. of oxygen) is 1.5 per

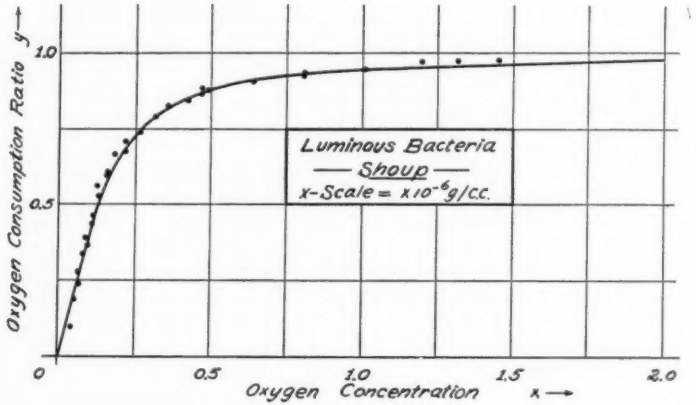

FIG. 2.

cent higher than at 2.3 cm Hg. of oxygen, the value given as the limit. This seems quite plausible since the experimental error throughout is of this order

of magnitude. The importance of having accurate values of the consumption for rather high oxygen pressures is indicated, from equation (67), it is seen that this is important if an accurate value of α is to be obtained.

In each of the two accompanying diagrams, the curve given is the theoretical curve. The experimental points are plotted. In the case of the Arabacia eggs, the values plotted were taken from "Tabulae Biologicae Periodicae" (Amberson, '28, Harvey, '32). The values plotted on the surve for the bacteria were taken from the curve given by Shoup ('29).

The solubility of oxygen was taken to be .028, so that we have 1 cm. Hg. of oxygen pressure equals $.53\times10^{-6}$ gm./cc. oxygen concentration (Wulff, '27).

DATA

A. Arbacia Eggs, Fertilized.
Amberson.

$\alpha = 7\times10^{-8}$ gm./cc.
$\beta = 5\times10^{-7}$ gm./cc.
$q_{3l} = 1.5\times10^{-5}$ gm./cc./min.
$r_0 = 3.8\times10^{-3}$ cm.
$c_{03}{}^* \sim 7\times10^{-7}$ gm./cc.

From which

$\dagger\begin{cases} D_3 \geq 3\times10^{-5} \text{ cm.}^2\text{/min.} \\ h_3 \geq 3\times10^{-2} \text{ cm./min.} \end{cases}$
$k \sim 1.5\times10^{-5}$ gm./cc./min.
$h_3 > 3\times10^{-2}$ cm./min.

B. Arbacia Eggs, Fertilized.
Tang and Gerard.

$\alpha = 2\times10^{-8}$ gm./cc.
$\beta = 18\times10^{-7}$ gm./cc.
$q_{3l} = 1.2\times10^{-5}$ gm./cc./min.
$r_0 = 3.8\times10^{-3}$ cm.
$c_{03}{}^* \sim 20\times10^{-7}$ gm./cc.

From which

$\dagger\begin{cases} D_3 \geq .6\times10^{-5} \text{ cm.}^2\text{/min.} \\ h_3 \geq .9\times10^{-2} \text{ cm./min.} \end{cases}$
$k \sim 10^{-5}$ gm./cc./min.
$h_3 > .8\times10^{-2}$ cm./min.

C. Arbacia Eggs, Unfertilized.
Tang.

$\alpha = 12\times10^{-8}$ gm./cc.
$\beta = 2.5\times10^{-7}$ gm./cc.
$q_{3l} = .35\times10^{-5}$ gm./cc./min.
$r_0 = 3.8\times10^{-3}$ cm.
$c_{03}{}^* \sim 6\times10^{-7}$ gm./cc.

From which

$\dagger\begin{cases} D_3 \geq 1.4\times10^{-5} \text{ cm.}^2\text{/min.} \\ h_3 \geq 1.8\times10^{-2} \text{ cm./min.} \end{cases}$
$k \sim .3\times10^{-5}$ gm./cc./min.
$h_3 > .7\times10^{-2}$ cm./min.

D. Bacteria, Luminous.
Shoup.

$\alpha = 4\times10^{-8}$ gm./cc.
$\beta = 1.8\times10^{-7}$ gm./cc.
$q_{3l} = 2.5\times10^{-2}$ gm./cc./min.
$r_0 = 7\times10^{-5}$ cm.
$c_{03}{}^* \sim 3\times10^{-7}$ gm./cc.

From which

$\dagger\begin{cases} D_3 \geq 4\times10^{-5} \text{ cm.}^2\text{/min.} \\ h_3 \geq 3 \text{ cm./min.} \end{cases}$
$k \sim 2\times10^{-2}$ gm./cc./min.
$h_3 > 2$ cm./min.

† One or both of the $\sim$ signs must hold.

V

Since it is only possible to estimate the value of the external concentration of lactic acid, CO_2, any estimates of the values of the coefficients of diffusion

and permeability for lactic acid are not very meaningful. Should experiments be performed with this in view, it is essential that the value of the external concentration of lactic acid to be used be maintained sufficiently long before beginning the experiment.

Experiments performed in which the external concentrations of glucose and lactic acid are small may perhaps need slight change due to actual depletion of the food supply of the cell during the experiment. This would be assumed to be proportional to the time integral of consumption rate of oxygen. Hence, if the experiment was performed beginning at high oxygen tensions, passing to low concentrations, then back to high values, it would be expected that the limiting value of the oxygen consumption would not return to the original value. The corrected curve would then be given by

$$q_3' = q_3(t) + g\int_0^t q_3(t)dt \tag{76}$$

or

$$y' = y(t) + g\int_0^t y(t)dt. \tag{77}$$

Since the total oxygen consumption is known, and the difference between y' and y at the end of the experiment is known, g is known. Also, from the total consumption of oxygen we have the net consumption of glucose kT. Hence if V = volume,

$$\frac{c_1V - kT}{c_1V} = \frac{y_T'}{y_T} \quad \text{and} \qquad kT = nq_{31}\int_0^T y(t)dt \tag{78}$$

we may estimate the value of c_1, the average value of the concentration of glucose. This value is obtained by other methods. Also, since T is the duration of the experiment in minutes, $kT/T = k$ which should approximate the value derived by equation (63), the values α and β being from the corrected curve. (The value of b, if known from equation (29) or (63), may also be taken into account in the correction.)

However, to be valid, preliminary experiments should be performed to determine the minimum concentrations to which the cells may be subjected without undue casualties. An estimation of the number of deaths as it varies with time would permit a similar slight correction as that due to depletion of glucose.

Experiments performed in flowing water of known concentration of oxygen would not include the possibility of oxidation of any matter during the experiments. Also c_{01} and c_{02}, could be made zero. Loss of lactic acid due to diffuson would be appreciable only at low tensions and the effect on the total loss of glucose throughout the experiment could be made slight. Then if experiments

were performed at various high values of c_{01} and c_{02}, some knowledge of the coefficients for glucose and lactic acid would be obtained.

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INTERPRETATION OF CERTAIN INFANTILE GROWTH CURVES

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The autocatalytic monomolecular reaction theory of growth proposed by Robertson (1908), whose operations are expressed by the sigmoid growth curve with the formula $\log y/A - y = K(t - t_1)$† is a very valuable conception. It certainly holds true for multiplication of unicellular organisms in a restricted medium. An attempt has been made to apply it to growth of the higher organisms, including man. It has been pointed out that the weight curve of human growth can be resolved into three curves, circumnatal, juvenile and adolescent, each in accord with the above formula (Robertson, '15, p. 41; Davenport, '26, p. 206) (Fig. 1). Our problem is, does a simple formula apply to the growth of single organs in a developing infant of the first post-natal year, and if not what additional considerations must be involked?

To throw light upon this problem measurements have been made of the development of different external dimensions of babies at frequent intervals during the first post-natal year. The result of such study upon a single organ, the arm, will be considered here.

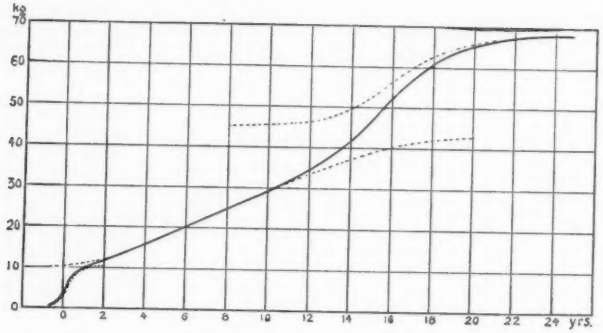
Fig. 2 shows the typical curves of growth in length of the proximal and distal segments of the arm of a particular baby, from birth to 10 months. One sees that the proximal segment (acromion to radiale, or *humerus*) is at birth the longer and grows more rapidly than the distal segment (radiale to stylion or *radius*) throughout the first months. Thus, the upper segment (humerus) at 5 to 6 months is increasing at the rate of 4 mm. a month, and continues at that rate for a time, declining to 20 at the end of the chart (10 months). The distal segment (radius) at 5 to 6 months is growing at the rate of 2.5 mm. per month and this rate falls to 1 mm. at the end of 10 months.

The ratio of the distal to proximal segment $\times 100$ is known as the "brachial index." At 2 months, in the case given, the brachial index is 92, at 10 months it is 80. This change in ratio is of interest in comparison with the brachial index at an earlier and in a later stage. On the average, in the third month of gestation the human brachial index is 74; at birth about 90 (Kugler, '32, pp. 474–476); on the average, in the adult, 78.‡ In anthropoid apes the brachial index similarly

* From the Carnegie Institution of Washington and Normal Child Development Study, Columbia University, Babies Hospital, New York City. Dr. Herbert B. Wilcox, Director; Dr. Myrtle B. McGraw, Associate Director. Constant cooperation of the staff of the Development Study is gratefully acknowledged.

† Where A is the final size attainable; t_1 the time at which the reaction is half completed; y the quantity of growth present at any given time, t. K is a constant depending on velocity of the growth, being the ratio of size gained to the size still to be gained divided by the distance in time from the axis of inflection.

‡ Schultz, '37, p. 301, from skeletal measurements, finds a mean of 75 for adult white males; he had previously found ('26, p. 489) an index of 83.6.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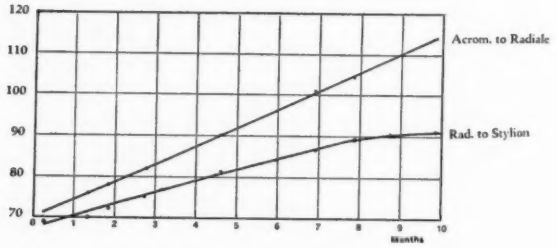
1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Acrom. to Radiale
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Months

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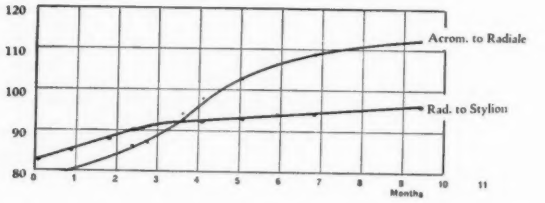
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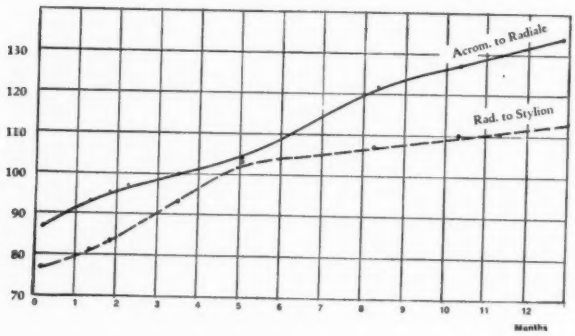
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4

rises during gestation to about 80 or 85 at birth (Schultz, '30, p. 363), but whereas in humans the brachial index thereafter diminishes, in the anthropoid apes it continues to rise, reaching 93 in the adult chimpanzee and 114 in the adult gibbon (Schultz, '37, p. 301).

There is considerable variation in the size of the maximal brachial index attained. Thus in Fig. 3 the baby acquires during the first post-natal month an index of 106, which reminds us of the Gibbon, but by 9.5 months in the same child the index is reduced to 86.

The age at which the maximum brachial index occurs is also variable. Fig. 4 gives the growth curves of the arm segments of an infant in whom the maximum brachial index is found at 5.5 months. In other cases the growth curves are so rapidly diverging and the brachial index is so low at birth that we think it probable that the maximum index must have occurred before birth.

The important thing is that in man, in contrast to the anthropoid apes, sooner or later usually somewhere in the circumnatal period the brachial index begins to decrease.

How do we account for this change in ratio? It is clearly due to changes in the relative velocity of growth in the two bones, the humerus and the radius. While the humerus continues to grow uniformly in much the anthropoid fashion, the radius has its growth inhibited.

If we look at the section of a growing long bone (Fig. 5) we shall find that it is increasing by the addition of materials at the two ends. The rate of growth then depends (1) upon the rate of cell division (which there is some reason for thinking is especially under the control of sulphur compounds); (2) upon the rate of water imbibition, probably due to lyophilic substances in the cell; and (3) upon the rate of production and secretion of collagen and elastin, which are the principal materials found in the formed substance between the cells. It is especially in determining the normal production of formed substance that vitamin D, or ergosterol, plays such an important part by preserving the normal form of the bone and preventing rickety growth. Sooner or later there are also growth promoting substances of endocrine origin which stimulate the growth processes. All these chemical processes, we must believe, are accelerated by the action of specific enzymes, which are probably genes, in the cells at the ends of the bone.

FIG. 1. Mass curve of growth in weight from birth to 24 years of male children of Nordic stock. Dotted lines represent the three assumed autocatalytic monomolecular constituent growth curves, in accordance with Robertson's theory. The present study is concerned only with the lower left hand corner of the diagram.

FIG. 2. Individual growth curves (smoothed) of humerus (acromion to radiale) above, and radius (radiale to stylion) below, from birth to 10 months. Joyce LaC. at Babies Hospital, New York City, Normal Child Development Study, No. 3409 ♀.

FIG. 3. Individual growth curves (smoothed) of humerus (above at right) and radius (below at right) from birth to 9.5 months, of Lionel LaJ. at Babies Hospital, Normal Child Development Study, No. 3301 ♂.

FIG. 4. Individual growth curves (smoothed) of humerus, (full line) and radius (broken line) during first 13 post-natal months of John P. McM. at Babies Hospital, Normal Child Development Study, No. 3411 ♂.

During the five months before birth the genes that are regulating growth processes at the ends of the radius are working with greater velocity than those at the ends of the humerus. This is true both of anthropoid apes and man. But whereas in anthropoids this differential growth continues to maturity (somewhat faster in the gibbon than in the chimpanzee) in Homo the growth rate of the radius becomes, shortly after birth, retarded, that is, partially inhibited more than in the anthropoid ape. This slowing up in the growth of the radius is probably not because of inherent inability of the radius to grow as fast, or for as long a time, after birth, as the humerus, for in the gibbon it continues to grow faster than the humerus. It would seem rather that a new inhibiting gene has become active in the human infant to slow down the growth of the radius.

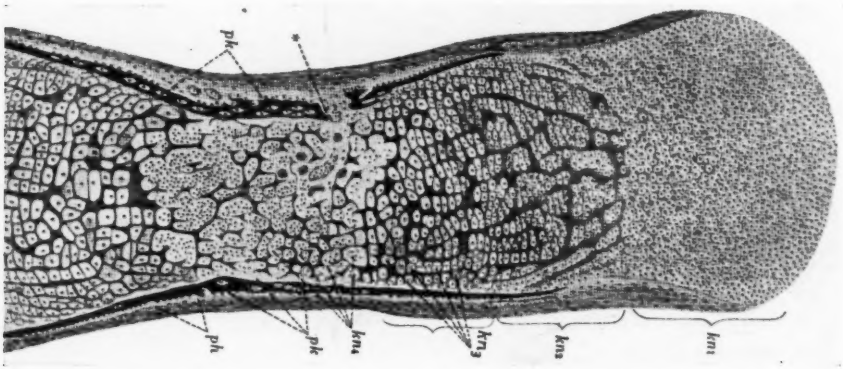

FIG. 5. Longitudinal section through long bone (proximal phalanx) of a three-months human embryo, showing at kn_1, unchanged cartilage; kn_2, columns of cartilage cells; kn_3, zone of calcifying cartilage; kn_4, vestiges of walls of broken down cartilage cavities; *pk*, perichondral (periosteal) bone; *ph*, perichondrium (periosteum);* connection between periosteum and the primary marrow. ×80. After Sobotta from Maximow-Bloom's *Textbook of Histology*, courtesy of W. B. Saunders Co., publishers.

In some way or other, the slowing down of the radius is tied up with an advantage to the human species. While in the tree-inhabiting gibbon a long forearm is essential in its flight-dance through the trees, in a surface-living, erect form, like man, it is a disadvantage.

The relative length of the distal segment of the arm, indeed, varies somewhat in different races of mankind, being relatively greater in Negroes than in Whites. And it is perhaps becoming, in the course of generations, less; but it is now just at about the right proportion for the application of force in the use of tools.

One concludes then that the growth of the radius slacks up to secure fitness to the needs of the species, or perhaps better, adult Homo Europeus finding himself with his low brachial ratio has successfully applied himself to tools. A special gene, or other mechanism, which causes the relative slowing down of cell proliferation and collagen formation at the ends of the radius has given Homo Sapiens a special advantage which has aided his survival.

This conclusion does not militate against the real value of Robertson's formula for some cases of growth even of human organs; but it is important to recognize that the general growth curve is a mass curve. It is compounded of more elementary individual growth curves that are not monomolecular in nature, but are the resultants of complex processes regulated to meet the developmental needs of the organism. Man is not comparable merely to a colony of unicellular organisms, but is rather an individual organism whose special parts are each controlled by laws of growth of their own, such as shall bring about the adequate fitness-to-meet-its-environment of the organism as a whole.

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DEVELOPMENTAL GROWTH AND l-PROLINE

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This is extension to the heterocyclic pyrrolidine compound proline of the inquiry into the part played in growth by the naturally occurring tissue components of general distribution.

The compound and its derivative oxy-proline are unique in that there is no $-NH_2$ group attached to the alpha carbon; instead the nitrogen is present as an $=NH$ group which unites the alpha carbon with the gamma.

Although this configuration is undoubtedly a significant contributor to the reaction basis of living (Vlés and Coulon '31: Hammett '37), it also is a direct participant in developmental growth as will be shown in the present report.

As usual the colonial-living form of the marine hydroid *Obelia geniculata* was the test animal. This and the experimental procedure have been described in papers references to which are given elsewhere in This Journal (Hammett and Schlumberger '37).

Like numbers of test and control animals of like distribution according to state of development at the beginning of observation were simultaneously grown in fresh sea-water cultures under like conditions of temperature, illumination, and pH. Four changes into fresh solutions were made during the 24-hour course of experiment.

Stock solutions containing 0.055 gram freshly prepared and pure l-proline in 100 cc. fresh sea-water provided daily the aliquots from which test culture solutions were made. The 1937 compound was prepared especially for this work by Dr. M. S. Dunn of the Amino Acid Manufactures, University of California, Los Angeles.

Eighty-two experiments over a concentration range from M/400,000 to M/8400 were run in two different seasons with two different sets of workers using close to 34,000 animals. Table 1 gives the raw data according to concentration and year together with the regression of the complete hydranths and the catabolic disintegration of the senile in terms of absolute percentage. Table 2 gives the direction of test deviation from control for each of the stated developmental and metabolic activities in each concentration and year. It is derived, constructed, and analyzed as described in detail in the l-aspartic acid report (Hammett and Schlumberger '37). Analysis will be made on the basis of the 1937 data since those of 1933 are less adequate.

RESULTS

As with other amino acids so with l-proline it is found there is an essentially ineffective concentration, a range of growth affecting concentration, and a

TABLE 1

THE NUMBER OF EXPERIMENTS, COLONIES, HYDRANTHS, AND GONOPHORES USED IN THE l-PROLINE STUDIES TOGETHER WITH THE PERCENTAGE REGRESSION AND CATABOLISM EXHIBITED BY TESTS AND CONTROLS AT STATED CONCENTRATIONS

M / 1000x	Year	No. Expt.	No. Colonies		No. Hydranths		No. Gonophores		% Regression		% Catabolism	
			Cont.	Test	Cont.	Test	Cont.	Test	Cont.	Test	Cont.	Test
400	1937	10	150	150	2341	2325	390	385	28.3	28.3	70.1	65.6
200	1933	3	38	38	426	427	60	54	27.6	35.7	90.6	92.9
200	1937	15	218	218	3378	3417	710	750	34.0	37.5	73.8	75.7
100	1933	8	113	113	1389	1387	107	86	29.4	38.5	88.7	89.0
100	1937	20	*266	267	3953	4007	763	752	21.5	30.6	80.8	79.0
50	1933*	2	24	24	281	281	28	24	43.2	63.6	92.4	95.0
50	1937	10	150	150	2458	2453	322	277	30.2	43.5	77.4	79.8
25	1937	10	150	150	2470	2467	181	207	42.3	60.9	86.8	88.8
16.7–8.4	1933†	4	48	48	439	597	40	40	42.7	68.0	92.9	93.6
Totals	—	82	1157	1158	17135	17361	2601	2575	—	—	—	—

* Includes one experiment at M/50,000 and one at M/25,000.

† Includes one experiment at M/16,700, one at M/12,500, one at M/10,000, and one at M/8400.

range of toxic concentration. The delimitation is as follows.

Since at M/400,000 there is no increase in test regression, while at M/200,000 such occurs; and since at M/400,000 test growth does not greatly deviate from control in 3 of the 4 developmental brackets, while at M/200,000 it distinctly

TABLE 2

TREND OF TEST DEVIATION FROM CONTROL WITH RESPECT TO DEVELOPMENTAL AND METABOLIC ACTIVITY IN l-PROLINE CULTURES

M / 1000x	Year	Initiatn.			Proliferatn.			Differentn.			Organizatn.			Maintn.			Catabol.		
		+	−	=	+	−	=	+	−	=	+	−	=	+	−	=	+	−	=
400	1937	0	2	5	4	3	1	2	3	4	1	2	5	4	5	1	4	6	0
200	1933	1	3	3	2	3	3	3	1	3	1	1	4	0	3	0	2	1	0
200	1937	3	0	4	2	4	2	5	3	2	2	2	4	5	9	1	6	8	1
100	1933	1	4	2	2	3	3	4	4	2	3	3	3	0	6	2	6	2	0
100	1937	3	3	1	2	5	1	4	1	3	1	0	6	1	17	2	9	10	1
50	1933	2	3	2	1	1	4	1	2	5	2	2	4	0	2	0	2	0	0
50	1937	0	7	0	2	5	1	4	1	4	2	1	5	0	10	0	6	3	1
25	1937	1	5	1	0	5	3	3	5	2	3	4	2	0	10	0	6	2	2
16.7–8.4	1933	0	5	2	2	3	2	1	4	4	0	3	5	0	4	0	3	0	1

deviates in 3 out of 4; the low M/400,000 concentration can be considered as essentially ineffective and hence omissible from further analysis.

Record is however made that here as elsewhere there is exhibited a trend towards inhibition of general growth in the lowest concentration, which is followed by no inhibition or relative enhancement at higher, with recurrence of inhibition at the highest (Hammett '37a). This will be discussed in detail in a later report.

Passing by the effective range it is seen that toxicity begins at M/50,000. The evidence is the trend to general growth retardation as contrasted with the absence thereof at the lower M/100,000 and M/200,000 concentrations. Thus—when the four developmental activities are combined—test growth is seen to have been less than control in 14 and greater in but 8 of its 32 expressions at M/50,000, while in its 33 expressions at M/100,000 it was less in but 9 and greater in 10. A retardation at M/50,000 is also shown in the 1933 data.

As would be expected toxicity increased with increase in concentration. This is shown in the 1937 data by the fact that whereas at M/50,000 test growth was less in 64 per cent of its deviations from control (using the data of the four developmental activities combined) it was less in 75 per cent at M/25,000. In the 1933 data this value increased from 65 per cent at M/50,000, through 75 per cent at the M/16,700–M/10,000 range, to 87 per cent at M/8400. Further the increase in greater test regression from 50 per cent at M/50,000 to 61 per cent at the M/16,700–M/10,000 range, with a subsequent drop to 51 per cent at the highest most toxic M/8400 concentration is supplementary evidence (Hammett '36a) as is the gradual decrease in relative catabolic activity of the tests over the same concentration increase (Hammett '36b).

Turning now to the non-toxic M/200,000–M/100,000 reactive range it is seen that the developmental activity of differentiation is expressed to greater degree in the l-proline than in the control cultures.

The evidence is that the change from the half to the three-quarter stage was greater than control in 9 and less in but 4 of the 20 expressions given by the 2 sets of 1937 experiments, and that when the data are combined test is greater than control in 5 and less in none of the 10 potentialities for differentiation activity expression. Further it tended to be greater in 3 and less in but 1 of the 5 concentrations studied. A like specific enhancement of differentiation was present in the 1933 M/200,000 concentration. This favoring was unique to differentiation.

Thus—test initiation expression was greater than control in 5 and less in 3 of the 14 expressions given by the 2 sets of 1937 experiments, and when the data are combined test is greater in 3 and less in 2 of the 10 potentialities for initiation activity expression. Though this is an apparent trend towards enhancement, it comes solely from the M/200,000 results and is not borne out either by the reaction at M/400,000 and M/100,000 or by the 1933 data. For as the table shows test initiation was less than control at M/400,000 and not greater at M/100,000 in 1937, while in 1933 it was decidedly less in both the M/200,000 and M/100,000 cultures. Initiation—if at all responsive to l-pro-

line—is sensitive to inhibition rather than to stimulation. This may well be secondary to the retardation of proliferation expression since for initiation to be made evident proliferation activity is essential.

Organization expression was unaffected. The evidence is that test change from the three-quarter stage to the complete functionable hydranth was greater than control in 3 and less in 2 of the 18 expressions given by the 2 sets of 1937 experiments, and that when the data are combined test is greater than control in one, less in one, and equal in 7 of the 9 potentialities for organization activity expression. Further in none of the 5 concentrations studied did test organization as a grouped developmental activity deviate significantly from control. A like unresponsiveness is shown in the 1933 results.

Proliferation on the other hand was retarded in the presence of l-proline. The evidence is that test change from the bud to half stage was less than control in 9 and greater in but 4 of the 16 expressions given by the 2 sets of 1937 experiments, and that when the data are combined test is weakly less than control in 4 and greater in but 1 of the 8 potentialities for proliferation expression activity. The 1933 data are not inconsistent.

M/200,000 l-proline determines an enhanced regression of the complete hydranths which increases with increase in concentration. Thus—whereas test regression was greater than control in 5 and less in 4 of the 10 experiments done at M/400,000, it was greater in 9 and less in but 5 of the 15 done at M/200,000, greater in 17 and less in but 1 of the 20 done at M/100,000, and greater in all of those done from M/50,000 on. Since regression was increased by l-proline in non-toxic concentration this is taken as evidence of a specific dynamic effect beginning at M/200,000 (Hammett '36b).

In the non-toxic concentrations catabolic disintegration of the senile animals is no whit different from the controls. Though a trend to increase in the higher range is seen, this was too small in degree to justify present analysis.

DISCUSSION

d-glutamic acid as well as l-proline acts to enhance differentiation expression in Obelia. That l-aspartic acid has a like influence is to be expected from the fact that it is the homolog of d-glutamic. The basis of the similarity is at hand. For glutamic acid is potentially capable of giving rise to proline through condensation to pyrrolidone carboxylic acid and reduction thereof to the pyrrolidine configuration (Mitchell and Hamilton '29). Conversely there is experimental evidence that proline may give rise to glutamic acid in tissue (Weil-Malherbe and Krebs '35). This apparent interconvertability of the two amino acids raises the question as to which is the developmental determinant.

There is, however, the possibility that neither of them as such is a direct participant; but that the differentiation significant group is to be found in a common intermediary. A known intermediary as noted above carries the pyrrolidone configuration (Mitchell and Hamilton '29). Interestingly enough, two other naturally occurring compounds which carry this in part, also have been

found to be stimulative of differentiation expression in Obelia; namely thymine (Hammett and Lavine '37) and cytosine (Hammett, Lavine and Lavine '37). Moreover cytosine also acts as a retardant of initiation just as do l-proline and d-glutamic acid. It hardly needs stating that l-hydroxy-proline like l-proline is a stimulus to differentiation expression. Exposition will be given in the next report.

These incontrovertible experimental findings give strong suggestion that in some part of the pyrrolidone configuration is to be found a specific chemical agent for differentiation.

Against this is the fact that no evidence of differentiation forwarding influence was found with uracil (Hammett '36d) which compound presumably carries the potentiality for development of the pyrrolidone configuration in part (Levene and Bass '31). But it is not impossible that the uracil used in the reported work was contaminated with other substances and it may well be that the intermediary metabolism of uracil does not give rise to the same configuration as that derived from glutamic acid, proline, cytosine, and thymine.

Ar any rate our data would seem to justify an examination of the so-called "organizer" by those who are engaged in the study thereof, for the presence, actual or derived, of a pyrrolidone configuration or part thereof.

Now St. Julian and Rose ('32) have stated that 50 mg. of proline daily fails to accelerate growth in weight of rats fed a proline poor diet. They conclude that proline is a dispensable amino acid. But our studies show that glutamic acid will produce the same forwarding of differentiation growth as proline. And glutamic acid is convertible into proline. And there is no record that the diet of the animals used by St. Julian and Rose was deficient in glutamic acid. Therefore it would appear as if there is yet no proof that proline is dispensable. To establish this it would seem necessary that rats be fed diets deficient in glutamic acid as well as proline, with readmission of each singly. But even here if adequate cytosine and thymine were present there might be selective anabolism—which is differentiation (Hammett '36)—and weight increase close to that of the controls on full diet.

Whether or not proline is an essential component of the diet has yet to be determined as perusal of Sure's reports ('24) and the comments of Mitchell and Hamilton ('29) thereon will show.

That l-proline is indispensable to growth is of course self-evident since it is an integral not an adventitious component of living protoplasm. Further evidence is the fact that although its influence is like that of d-glutamic acid in some respects it differs in others and in sufficient diversity to maintain the postulate that each amino acid has its own specific and peculiar role to play in developmental growth (Hammett '37d). Thus l-proline has a distinct retarding influence on proliferation while no such effect is shown with d-glutamic acid. Further—regression begins with d-glutamic acid at M/400,000; but does not appear with l-proline until M/200,000 concentration is used. Finally l-proline is toxic to Obelia growth at M/50,000 while d-glutamic acid if toxic at all is not so save at the high M/6250 level.

SUMMARY AND CONCLUSION

Eighty-two experiments with close to 34,000 Obelia hydranths, half of which were exposed to l-proline in different concentrations, show that the amino acid is a specific participant in some process concerned with differentiation expression; that it acts as a retardant of proliferation and secondarily of initiation; that it has no apparent effect on organization; that it has a specific dynamic action; that it has no great influence on catabolism; and that it is toxic at M/50,000.

The findings when correlated with other data suggest that a specific chemical agent for differentiation may be found in some part of the pyrrolidone configuration.

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l-HYDROXYPROLINE IN DEVELOPMENTAL GROWTH

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For some reason or other l-hydroxyproline has received little attention although discovered in 1902 by Fischer.

The neglect may lie in the assumption that since the compound is apparently derived from l-proline with ease both without and within the living organism (Richter '23: Sporer and Kapfhammer '30; Adelene '31), and since its specific dynamic effect is negligible (Rapport & Beard '27), its significance is also negligible.

The results herein reported demonstrate that the compound is not the inactive tissue component early reports would have us believe.

As usual the colonial-living form of the marine hydroid *Obelia geniculata* was the test animal. This and the experimental procedure have been described with references elsewhere in This Journal (Hammett and Schlumberger '37).

Like numbers of test and control animals of like distribution according to state of development at beginning of observation were simultaneously grown in fresh sea-water cultures under like conditions of temperature, illumination, and pH. Four changes into fresh solutions were made during the 24-hour course of experiment.

Stock solutions containing 0.066 gram freshly prepared pure l-hydroxyproline in 100cc. fresh sea-water provided daily the aliquots for the test culture solutions. The 1937 compound was made especially for this work by Dr. M. S. Dunn of the University of California, Los Angeles.

Eighty-six experiments over a concentration range from M/400,000 to M/6250 were run in two different seasons with two different sets of workers using close to 41,000 animals. Table 1 gives the raw data according to concentration and year together with the regression of the complete hydranths and catabolism of the senile in terms of absolute percentage. Table 2 gives the direction of test deviation from control for each of the stated developmental and metabolic activities in each concentration and year. It is derived, constructed, and analyzed as described earlier (Hammett and Schlumberger '37). Analysis here will be made on the basis of the 1937 data since those of 1933 are less adequate.

RESULTS

There was a shift from a state of growth enhancement at M/50,000 to a state of retardation trend at M/25,000. The evidence from the 4 developmental activities combined is that test growth was greater than control in 14 and less in

but 10 of its 34 expressions at M/50,000, while at M/25,000 it was greater in but 12 and less in 14. Further, in the 1933 data test growth was greater than control in 10 and less in but 7 of the 32 expressions at M/50,000, while of the 34 at M/25,000 it was less in 10 and greater in but 7.

Overt toxic action however, was not apparent until M/12,500. Here test growth was less than control in 2 and greater in none of the 4 developmental activities, and when these are combined it was less in 14 and greater in but 7 of the 34 expressions.

Although the slight difference between test and control growth at M/25,000

TABLE 1

THE NUMBER OF EXPERIMENTS, COLONIES, HYDRANTHS, AND GONOPHORES USED IN THE l-HYDROXYPROLINE STUDIES TOGETHER WITH THE PERCENTAGE REGRESSION AND CATABOLISM EXHIBITED BY TESTS AND CONTROLS AT STATED CONCENTRATIONS

M / 1000x	Year	No. Expt.	No. Colonies		No. Hyranths		No. Gonophores		% Regressn.		% Catabolism	
			Cont.	Test	Cont.	Test	Cont.	Test	Cont.	Test	Cont.	Test
400	1937	10	150	150	2551	2568	142	161	24.6	24.4	79.2	80.4
200	1937	12	174	174	2940	2942	156	168	26.9	29.3	70.4	69.5
100	1933	3	46	46	624	624	43	34	38.8	36.4	94.5	94.9
100	1937	12	170	170	3095	3098	183	187	25.8	28.0	67.0	70.7
50	1933	6	91	91	1307	1307	92	112	31.8	33.4	91.4	91.0
50	1937	15	223	223	3541	3551	263	271	40.3	42.6	80.7	79.6
25	1933	3	45	45	666	674	73	33	33.0	28.5	95.7	91.6
25	1937	10	149	150	2301	2336	191	194	40.5	39.9	86.6	85.8
12.5	1937	10	150	150	2391	2400	211	243	36.8	37.9	88.4	83.5
6.25	1937	5	74	74	1198	1206	98	93	27.0	35.5	87.7	73.2
Totals	—	86	1272	1273	20620	20706	1452	1496	—	—	—	—

as contrasted with enhancement at M/50,000 and retardation at M/12,500 might indicate that this was a neutral instead of a toxic zone, yet since the data do indicate a shift thereat, it seems logical that it should be excluded from particulate analysis. This will therefore be based on the data of the M/400,000 to M/50,000 range inclusive.

Note is made of the fact that even at the low M/400,000 level growth response to l-hydroxyprlone is in evidence. Thus of the 30 expressions of the combined data of the 4 activities test growth was greater than control in 12 and less in but 8.

Turning to the separate developmental activities it is seen that initiation expression was retarded in the presence of l-hydroxyproline. The evidence is that it was less in 2 of the 4 sets of experiments and greater in none; that it was less in 11 and greater in but 6 of the 28 expressions presented by the 4 sets of experi-

ments; and that it was less in 3 and greater in but 1 of the 7 potentiality types when each is combined into a single value for the 49 experiments. As might have been expected the sensitivity extended into the neutral and toxic zones. Taking the data as a whole the trend of test initiation was less than control in 6 of the 7 7 concentrations. The 1933 data are not inconsistent.

Proliferation expression was enhanced in the presence of this amino acid. Thus it was greater than control in 3 and less in none of the 4 sets of experiments; it was greater in 15 and less in but 7 of the 32 expressions given by the 4 sets of experiments; and it was greater in 4 and less in but 1 of the 8 potentiality

TABLE 2

TREND OF TEST DEVIATION FROM CONTROL WITH RESPECT TO DEVELOPMENTAL AND METABOLIC ACTIVITY IN l-HYDROXYPROLINE CULTURES

M / 1000x	Year	Initiatn.			Proliferatn.			Differentiatn.			Organizatn.			Maintn.			Catabol.		
		+	−	=	+	−	=	+	−	=	+	−	=	+	−	=	+	−	=
400	1937	3	3	1	4	1	3	4	1	3	1	3	3	4	5	1	6	4	0
200	1937	1	3	3	4	1	3	3	4	3	2	4	3	1	10	1	5	6	1
100	1933	2	3	2	3	1	3	3	2	4	1	2	5	2	1	0	2	1	0
100	1937	1	3	3	4	1	3	6	1	3	1	3	5	2	9	1	5	6	1
50	1933	3	3	1	0	2	6	5	1	3	1	1	6	2	3	1	3	2	1
50	1937	1	2	4	3	4	1	5	2	3	5	2	2	6	7	2	5	9	1
25	1933	2	4	1	3	2	3	1	2	7	1	2	6	3	0	0	0	3	0
25	1937	2	3	2	4	3	1	3	6	1	3	2	4	5	4	1	4	5	1
12.5	1937	1	2	4	2	4	2	4	3	3	0	5	4	3	6	1	2	7	1
6.25	1937	1	6	0	3	2	3	3	2	5	5	1	2	0	4	1	1	4	0

types when each is combined into a single value for the 49 experiments. As might not have been anticipated a trend to like response was had in the neutral and high M/6250 concentrations so that in the 7 sets test proliferation was greater in 5 and less in but 2. The 1933 data are consistent.

Differentiation expression also was enhanced. Thus test was greater than control in 3 and less in none of the 4 sets of experiments; it was greater in 18 and less in but 8 of the 38 expressions afforded by the combined data; and it was greater in 6 and less in but 2 of the 10 potentiality types when each is combined into a single value for the 49 experiments. Not to have been expected was extension of the trend into the toxic zone so that test differentiation expression was greater than control in 5 and less in but 2 of the 7 concentrations. The 1933 data show increased differentiation at M/50,000 l-hydroxyproline.

Test organization expression tended to be less than control. It was less in 3 and greater in but 1 of the 4 sets of experiments; it was less in 12 and greater in but 9 of the 34 expressions afforded thereby; and it was less in 2 and greater

in none of the 9 potentiality types when each is combined into a single value for the 49 experiments. The 1933 data show the same trend. The response in toxic concentration is complicated by increased regression at M/6250 (Hammett '36).

Although regression tended to be greater in test than control in the majority of experiments, viz., in 31 as against 13 being less in the total of 49, the degree of difference was quite small, not only in each individual experiment but also in the data combined according to concentration. The plurality was unrelated to concentration. It was marked at M/200,000 and M/100,000 but insignificant not only at M/400,000 but also at M/50,000. Low grade reaction was also present in 1933. And in the toxic range until M/6250. The picture as a whole indicates that the complete hydranths are not particularly sensitive to l-hydroxyproline.

Catabolism expression was not particularly responsive. It was greater in the tests in 21 of the 49 experiments and less in 25; it was greater in 1, less in 1, and equal in 2 of the 4 sets of experiments; and when the data are combined according to concentration the difference in percentage is small and inconsistent in direction. The usual decrease with increase in toxicity was exhibited (Hammett '36a).

DISCUSSION

The substitution in l-proline of an OH in position 3 makes of l-hydroxyproline a compound which has quite a different effect on *Obelia* than the pyrrolidine carboxylic acid (Hammett and Collings '37).

It is less toxic as shown by the fact this effect is not open until M/12,500 while with l-proline it is present at M/50,000.

The compound has little if any effect on regression, little if any specific dynamic action (Hammett '36a), a finding which agrees with the report of Rapport and Beard ('27). l-proline on the other hand has a definite specific dynamic effect and produces marked increase in regression which increases with increase in concentration up to the highly toxic M/6250 level whereat it lessens as usual (Hammett '36a).

The differentiation forwarding effect of l-hydroxyproline appears at M/400,000. With l-proline this does not occur until M/200,000.

And finally l-hydroxyproline stimulates proliferation expression while l-proline has no such action.

Now l-tyrosine is less toxic than l-phenylalanine and has a greater enhancing effect on differentiation (Hammett '36a). And l-tyrosine is l-phenylalanine in which OH has been substituted for H in the para position of the benzene ring.

Further thymine is less toxic than cytosine and has a greater enhancing influence on both differentiation and proliferation (Hammett and Lavine '37: Hammett, Lavine, and Lavine '37). And thymine differs from cytosine not only by having a CH_3 in position 5 but also by having a potential OH substituted for NH_2 in position 6.

Thus in three instances it is shown that the OH group in these organic combinations has properties of its own in growth and toxicity.

It will be interesting to see if they are similarly exhibited in l-serine and d-beta-hydroxyglutamic acid when such become available.

Four possibilities exist.

The OH as such may have a direct influence.

Its presence in the molecule may produce that structural configuration and activity state therein which is essential to influence, and which with OH absent is not attained until the compound is subjected to intermediary metabolism. This would mean the difference between immediate and delayed action.

It may be that the presence of OH in the molecule favors the formation of the effective configuration in intermediary metabolism.

Or it may be the OH compound is a first step in the production of an effective configuration in intermediary metabolism and hence when this is used initially delay in the production of the latter is lessened.

The absence of answer to these questions does not lessen the importance of the correlation.

The enhancement of proliferation expression by l-hydroxyproline merits analysis not only in its own right but also as a step in tracking down the chemical group concerned in differentiation.

Since the growth response to l-hydroxyproline was more like that to thymine —and to cytosine somewhat—than it was to l-tyrosine it is clear that the factor is more closely related to heterocyclic pyrrole and pyrimidine than to carbocyclic benzene.

Since introduction of OH does not endow l-tyrosine with the property of enhancing proliferation expression, and since cytosine tends to have this effect without extra OH, it is probable that OH as such is not the immediate factor in proliferation response.

Now neither d-glutamic acid, l-aspartic acid, nor l-proline which act like l-hydroxyproline, cytosine, and thymine in forwarding differentiation, have any forwarding effect on proliferation. Yet these amino acids are interrelated through pyrrole derivatives (Mitchell and Hamilton '29) and presumably not through pyrimidine. It therefore may be assumed until the contrary is established that the proliferation forwarding action of l-hydroxyproline does not reside in its pyrrole relationship.

But it has been shown that uracil has a definite forwarding effect on the proliferation phase of animals derived by recurrence (Hammett '36b). And thymine is more closely related to uracil than is cytosine (Levene and Bass '31). Indeed it is more closely related to uracil than to cytosine. It is therefore probable that the greater enhancement of proliferation by thymine as contrasted with cytosine is *not* due to the acquisition of a potential OH but the closeness of its structural and chemical relationship to uracil. The proliferation forwarding property of cytosine is traceable to its metabolic transformation to uracil (Hammett '36b).

The hypothesis may therefore be tentatively advanced that the introduction of OH into l-proline determines the production therefrom in intermediary metabolism of a proliferation affecting configuration similar to that derived from

uracil. As pointed out elsewhere the proliferation effect of uracil is probably indirect through sulfhydryl (Hammett '36b).

This implies that the breakdown of l-proline differs from that of l-hydroxyproline since the former gives no proliferation forwarding effect nor derivative. Consistent with this implication is the demonstration by Knoop *et al* ('36) that beta-hydroxy-alpha-amino acids are not broken down in the same manner as the simple amino acids.

Now the pyrimidine uracil showed no distinctive differentiation effect and the response to cytosine and thymine was no greater if as great as that exhibited by the pyrrole derivative potential compounds d-glutamic acid, l-proline, l-hydroxyproline, and l-tyrosine when taken as a group.

It can therefore be assumed until the contrary is established that the differentiation forwarding configuration is more directly allied to pyrrole than to pyrimidine. Or more directly derived therefrom.

As pointed out in the preceding report (Hammett and Collings '37) pyrrolidone carboxylic acid is a probable interrelating compound of glutamic acid and proline.

Inspection of its formula in comparison with that of cytosine and thymine shows there is a correspondence in the presence in each of a carbonyl-imidemethine or C—N—C sequence.

O H H

Whether or not this particular sequence is the point of departure for a common differentiation forwarding configuration is of course unknown. Its presence however is suggestive enough to justify the postulation as a working hypothesis.

Of course it may be that the pyrrole derivative producing compounds act on some phase of differentiation other than that affected by the pyrimidine. For it is certain that more than one process with more than one set of determinants, participants and reactions is concerned in the developmental activity of differentiation (Hammett '36c). But the correlations here noted unavoidably evoke the possibility of a common derivative.

The prediction is made that in the pyrrolidone configuration or some part or derivative thereof there will be found that chemical grouping which is essential and specific to some phase of differentiation—that chemical specialization of the cells which brings them to functionable and structural maturity.

This is as good a place as any to point out that our ignorance of the intermediary metabolism of the pyrimidines is colossal. The entire subject needs intensive and broad investigation. Here is a rich and essentially untilled field for one seeking a problem.

It is commonly believed that hydroxyproline is not an essential amino acid (Sporer and Kapfhammer '30: St. Julian and Rose '32) since it may be derived from proline (Adelene '31). It seems necessary to state and to emphasize the thesis that each and every integral chemical component of living tissue of gen-

eral distribution is essential to the formation and growth of that tissue (Hammett '37) else it would not be there, and that even if animals increase in weight when a given component is absent from the diet, this is no proof the compound is not essential to some growth process (Hammett '37a).

A few words concerning the relation of these findings to cancer is in order.

Cancer is a growth and as such it should be studied. It has two chief characteristics—proliferation unusual for the site and time—and an incompleteness of differentiation.

It would seem desirable to retard the cell increase in number and to speed up the differentiation if this can be done.

The compounds or groups which nature uses to do these things in the normal course of development of an organism to maturity should be the ones best suited for putting a brake on neoplastic growth.

Already evidence has been given that proliferation can be slowed down by such a chemical group (Staff of The Lankenau Hospital Research Institute '36).

It is possible that in this group of differentiation forwarding compounds described in this series of papers there may be something which will bring cancer cells closer to maturity and hence delay its lethal action.

l-hydroxyproline would hardly be first tried because it favors proliferation as well as differentiation. But l-proline should be an ideal substance to test the hypothesis in relation to mouse and other tumors. For this amino acid not only forwards differentiation, it also acts to retard proliferation. What is more the compound is easily soluble and is neutral in reaction in solution. All that is lacking is a plentiful supply for adequate testing. To state that the compound must be of unquestioned purity is to state the obvious.

SUMMARY AND CONCLUSION

From 86 experiments done by two sets of workers in two different seasons with close to 41,000 animals half of which were exposed to l-hydroxyproline in concentrations ranging from M/400,000 to M/6250 it was found that the compound definitely accelerates proliferation and differentiation expression in *Obelia geniculata*.

Both initiation and organization expression tended to be retarded.

The compound has no marked influence on the changes associated with maintenance, regression, or specific dynamic action. It may induce a slight trend to a lessened catabolism.

Correlating these data with those previously reported on other amino acids and nucleic acid derivatives it is postulated that the pyrrolidone group or some part or derivative thereof is specific as a determinant and participant in some process essential to differentiation.

The implication of these findings to nutrition and cancer is pointed out.

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LOCALIZATION OF –SH AND –S. S– IN *OBELIA GENICULATA*

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In a report of the isolation and identification of glutathione Hopkins ('21) noted in passing that a positive nitroprusside test is given in root-tips and gave credit to Gola ('02) for first making this observation. The inference is that the compound is glutathione.

Following Hopkins, Shearer, reported glutathione in eggs and sperm of E. Miliaris ('22); Murray ('26) and Yaoi ('28) demonstrated both the reduced and the oxidized form in chick embryos and noted changes with age and growth; Denny ('26) showed that thio- compounds accelerated sprouting of potato tuber; and Baker ('29) indicated that glutathione was helpful in tissue cultures.

The significance of these associations was established by Hammett ('29) who demonstrated that the substance present in mitotically active tissue responsible for the nitroprusside test is largely sulfhydryl, and that this group acts as a specific stimulus to cell increase in number. A reference list is to be found elsewhere in a review (Hammett '35).

It was shown that the proliferation acceleration was exhibited in embryonic, reproductive, normal, pathological, replacement, repair, and regenerative tissues in studies made on plants, paramecium, hydrozoa, annelids, arthropods, molluscs, pisces, rodents, and man.

The conclusion was that sulfhydryl is a naturally occurring chemical group essential to some phase of cell multiplication.

Following Hammett many workers have confirmed the observations on –SH and proliferation (Sharpe '30, Vivario and Lecloux '30, Voegtlin and Chalkley '30; Binet and Arnaudet '31, Dulzetto '31, Rapkine '31, Wilkerson and Gortner '32; Coldwater '33, Gregory and Goss '33, Guthrie '33, Jahn '33, White '33; Zirpolo '35; Berthelot and Amoureux '36; Voegtlin, Johnson, and Thompson '35; Chalkley '37).

This paper extends to *Obelia geniculata* the study of correlation between growth, metabolism, and sulfhydryl.

MATERIAL AND METHOD

Colonies of *Obelia geniculata* were collected intact in fresh seawater on *Balanus* and *Mytilus* with care to prevent injury. Single colonies of 5 to 15 animals were freshly cut and transferred by a wide mouth medicine dropper to glass slides with a minimum of fluid. Examination under the microscope elim-

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inated imperfect colonies and colonies containing red pigmented material in the gastro-vascular cavity. The latter were found unsuited for color tests because of difficulty in discriminating between ingested matter and color resulting from the nitroprusside test.

Colonies were selected carrying the desired growth stages as described by Hammett ('33, '35, '35a) and after excess seawater had been removed the nitroprusside test was made as described below. Examination was made with a 10× eyepiece and 8× .2na and 40× .65na dry objective with Abbe condenser and blue color filter. Cover glasses were found unnecessary save when high power examination was made: then these were supported by thin pieces of cover glass.

It is necessary to use animals in active growth. Animals collected from warm summer waters do not give as good or as regular reactions as do animals taken from cold fall and winter waters and observed after growth has been stimulated by transfer to a warm laboratory.

The nitroprusside test for free −SH was used according to the modified Hopkins method described by Joyet-Lavergne ('28) and Giroud and Bulliard ('33) controlled according to the requirements set forth in another place (Hammett and Chapman '37) viz., a drop (.02 cc.) of supersaturated $(NH_4)_2SO_4$—or sufficient solid crystals to insure saturation; 2 drops (.04 cc.) 1% ammoniacal sodium-nitroprusside (.02 cc. NH_4OH per 10 cc. solution); and 1 to 2 drops (.02–.04 cc.) Aq. NH_4OH.

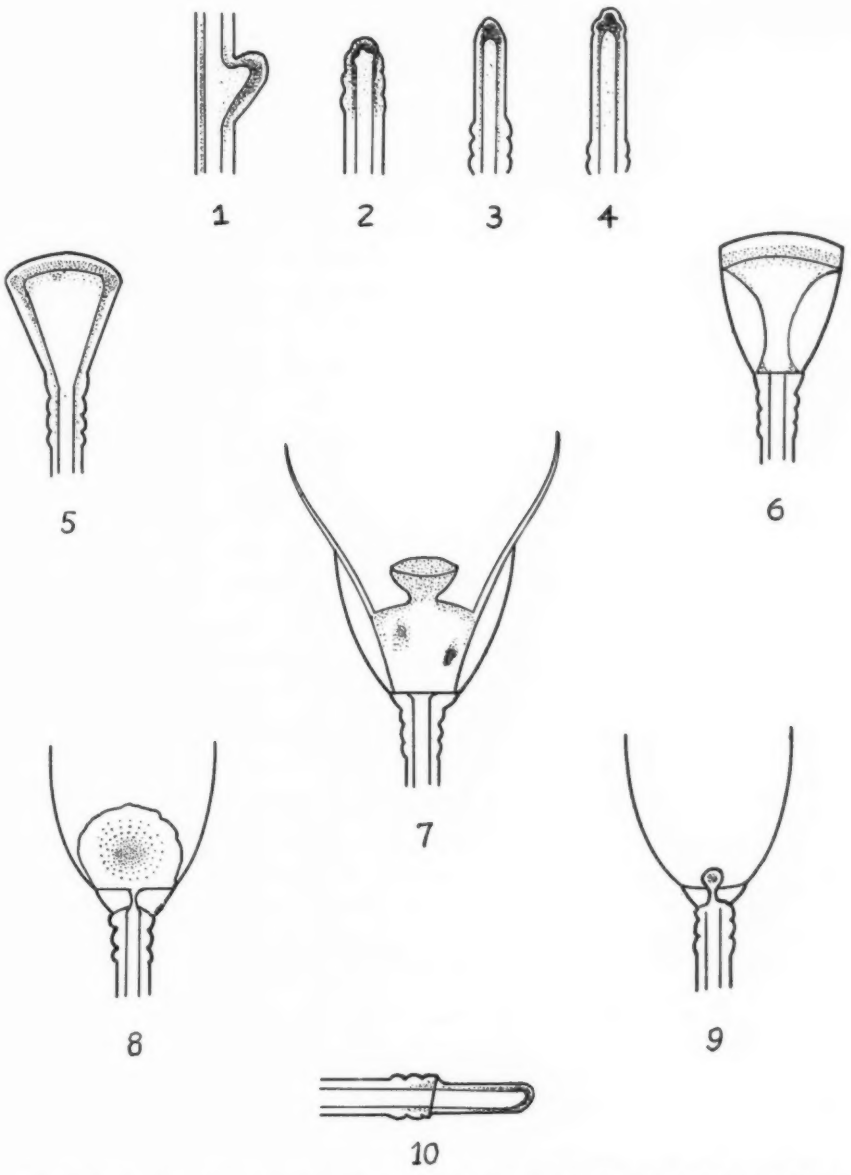
Disulfide was demonstrated by preliminary exposure of the colony to 2 drops (.04 cc.) of 2% KCN solution for 10 minutes. Excess was removed by micropipette and 2 drops of 1% ammoniacal sodium-nitroprusside added. The color is pink to violet. It is enhanced by saturated $(NH_4)_2SO_4$. NH_4OH is unnecessary since sufficient −OH is provided by KCN.

Care must be taken to not use colonies showing red or pink on addition of any reagent prior to nitroprusside. Sulfate particularly so acts on some granular material occasionally found scattered throughout *Obelia:* possibly red algae detritus.

RESULTS

The outstanding observation is that −SH reactions are generally found in varying intensities in proliferating regions of the developing hydranths. Regressing hydranths show a diffuse reaction in the disintegrating mass and functionally mature animals give one in the gastro-vascular cavity.

R−SH was notably localized in the proliferating buds (Figs. 1, 2, 3, 4). In young buds or bud primordia (Fig. 1) which are indicated only by bulging coenosarc and perisarc, a red-violet reaction belt appears in the endoderm around the hydrocaulus throughout the adjacent region—more intense at point of origin of the bud than on the opposite side. As the buds develop the reaction recedes away from the hydrocaulus (Fig. 2) and becomes localized along the forming pedicel and then in the thick endodermal cells at the distal end of the bud. From this reacting cap of cells there is a steadily diminishing



Localization of free −SH in growing parts of *Obelia Geniculata*. 1-2-3-4-Buds: 5-Halfstage: 6-Three-Quarterstage: 7-Complete Hydranth—note food particles highly stained for reduced −S.S−: 8 Senile—note inner localization: 9-Goblet: 10-Stolon.

color extending back along the endoderm, but it rarely extends beyond the endoderm of the proximal annulations. The same is seen in active main-stalk buds whether these possess terminal annulations (Fig. 4) or not (Fig. 3).

After KCN, which shows R−S.S−R as well as R−SH, the same localization appears as would be expected, and in addition a reaction in the distal ectodermal layer. Occasionally the bud hydroplasm may show a faint hint of −SH and −S.S− but this is rare. When it does appear it is snuckup against the inner side of the distal endoderm.

A good test for −SH and −S.S− is also given in so-called "Half" stage (Fig. 5) of hydranth development. This stage is characterized by a cone-like expansion at the distal end of the hydrocoele with ectoderm underlaid by endoderm directly contacting the chitinous layer of the perisarc throughout (Nutting '15). The becoming of this stage is one of proliferative growth with but little differentiation. The −SH reaction is concentrated in the thick endodermal layer at the base of the cone and diminishes sharply along the same layer down the side. Occasionally in very young Halves the reaction extends into the endoderm of the proximal annulations. The ectoderm apparently contains −S.S− for it gives a faint pink nitroprusside test after reduction. In the hydroplasm also some such reaction is occasionally given and as with the bud the nearer the growing part the deeper the color. Sometimes the ectoderm may show R−SH without reduction but infrequently.

Free sulfhydryl is also present in the "Three-quarter" developed hydranth but to a much less degree. In the becoming of this stage proliferative growth still occurs but the chief growth activity is differentiation. As usual the −SH is concentrated in the endodermal layer of the distal cell mass. It may extend slightly into the endodermal cells of what is to be the gastro-vascular cavity. Occasionally there is a slight test in the differentiating region of the diaphragm.

When rudiments of tentacles are forming as made evident by a ridge of endodermal cells which push out the overlying ectoderm there may be a strong reaction for disulfide. Disulfide is usually present in the ectoderm of the cap. Tentacle buds show a concentrated reaction. The hydroplasm bordering the distal endodermal cap frequently shows after cyanide a definite localization while the hydroplasm in other regions within the animal gives a faint and generally diffuse tint. The thin layer of ectodermal cells which is to contribute to the walls of the stomach gives no reaction except in regions adjacent to budding tentacles and in regions of the hydranth diaphragm.

Note is made that $(NH_4)_2SO_4$ brings out the structural composition of the Three-quarter stage.

In the mature animal (Fig. 7) characterized by functioning stomach, mouth and tentacles and a free chitinous hydrotheca, diffuse reaction is always found in the mouth and gastro-vascular cavity. Occasionally food particles and organisms can be seen in the latter, which react more vividly than the surroundings. Brown granules may obstruct color view.

Although tentacles adjacent or in contact with the mouth may appear to give a test, careful focussing shows the entire tentacle non-reactive and the

appearance merely due to color transmitted through the transparent structure. Food particles and organisms found near or adjacent to the tentacles may give the test.

In regressing or senile animals there is lost the definite tissue localization of −SH evident in the developing organism (Fig. 8). Instead there is a diffuse but definite coloration throughout the central mass. In the seniles the test for disulfide is more intense in the center than at the periphery.

When the so-called "Goblet" stage is reached (Fig. 9) only a small pea-like mass remains on a small stalk and the hydroplasm is still continuous with that of the hydrocaulus. This small mass of material still gives a faint reaction for sulfhydryl. An empty hydrotheca which contains no degenerating material gives no test. However, buds growing by proliferation from the terminal annulation of the hydrocaulus of the old goblet show the usual reaction for buds already described.

Stolons formed from cut ends of colonies kept in fresh seawater show −SH in endoderm and −S.S− in the ectoderm (Fig. 10). The main colonial hydrocaulus may also show a faint test localized in the endoderm proximal to the former cut end.

Sulfhydryl is liberated from the hydrocaulus by injury just as it is from roots (Hammett '29) for when the stalk is cut or scratched in the presence of the reagents a faint pink flash can be seen.

Although it is the disulfide group which is usually found in the ectoderm and the sulfhydryl in the endoderm, it is true that occasionally sulfhydryl is found in the ectoderm as well as in the endoderm.

In certain colonies, but not all, −S.S− is generally distributed in both ectoderm and endoderm in non-growing parts. Despite this diffuse reaction the localization of intensity in growing parts is as described above.

The chart only shows −SH: superfluous would be −S.S− addition.

DISCUSSION

That the nitroprusside test as rigidly applied in this experiment is a test for free sulfhydryl has been shown (Hammett and Chapman '37). Preliminary exposure to KCN and subsequent application of the test gives not only the −SH originally present but also that liberated by reduction of disulfide. Discussion of the test in relation to denaturization studies of Mirsky ('37) has been adequately given by Chalkley ('37).

Apparently permeability plays no significant role in the localization of the S-groupings in *Obelia* for in the bud, half, and three-quarter stages and in the stolon—all of which possess a chitinous coat throughout—the reaction is circumscribed while in the complete hydranth, and in senile and goblet stages in which tissue is directly exposed to chemical action the reaction is diffuse.

The fact that sulfhydryl is found diffuse within the gastro-vascular cavity and disulfide concentrated around solid matter contained therein allows the conclusion without fear of contradiction that it is here that food for the colony is being digested. That −SH is also localized within what is called the mouth

would indicate that on this surface or within these cells digestion also occurs. Perhaps it is the cells of the mouth which produce the proteolytic enzymes of *Obelia* and perhaps digestion starts on this surface to be continued in the gastrovascular cavity. Here is a nice piece of work to be done. But regardless of the exact origin of the digesting enzymes or the exact localization of digestion it is quite clear that *Obelia* eats by ingestion and not by absorption; that it has a digestive alimentary system of which that of animals much higher in the scale of evolution are but prototypes; and that it can well be included in the list of animals suitable for nutrition and other studies dealing with intermediary metabolism.

The frequent detection of disulfide in the streaming hydroplasm indicating as it does the presence of an S-containing amino acid suggests that in *Obelia* as in higher forms tissue bausteine are provided by intermediary metabolism for distribution in simple rather than in complex form. Consistent with this is the general presence of free −SH in decent concentration apparently in the hydroplasm just adjacent to the distal growing regions. While it is possible this localization is from transmitted color through transparent tissue in some cases, and while it is possible that in others the reacting −SH may have come into the hydroplasm by diffusion from the active distal endoderm, yet the impression persists that there is in the distal hydroplasmic stream at times a real concentration of this chemical group. Such a finding could be possible as evident from the fact that the stream is towards the growing part (Hammett and Padis '35) and from the fact easily verifiable by observation that around many of the granules which may be seen being hurried to the growing part in the hydroplasm there may be frequently obtained a positive test for disulfide.

Both sulfhydryl and disulfide are found diffuse within the core of the senile animals but concentrated within instead of at the periphery. This justifies the conclusion that substance is undergoing chemical dissolution and that this is from within. This then is chemical proof *in vivo* of Hammett's ('35a) contention that the gradual disappearance of the senile animal is an endogenous process and a mark of catabolic activity.

Turning now to the correlation between sulfhydryl localization and growth it is seen that wherever growth is active −SH is present in the endoderm; and that where there is no growth in evidence there is no −SH in the cells.

The data show that as −SH follows the development of the hydranth from its anlagen on the hydrocaulus to the complete organism it remains at its highest intensity in just those regions where cell increase in number is taking place for the formation of new and additional tissue. It is significant that it is most diffuse where the preparatory proliferations are taking place incident to the production of a new growth, and concentrated right in the location wherefrom that new growth is springing. That as this proceeds to form a bud and grow a pedicel, the intensity of concentration increases in the most distal most rapidly growing part and diminishes proximally therefrom to leave entirely the region of origin in the hydrocaulus; but still staying and weakly somewhat within the new growing annulations. That as the bud expands into the inverted cone of the half, the concentration intensity is still strong in the distal cell layer but

perhaps not quite so strong as in the bud and weakens out in the proximal organismic endoderm only rarely to extend down into the annulations. And that as the cone differentiates into the three-quarter truncated cylinder the intensity concentration seems less and the localization is more restricted being almost entirely within the endoderm of the head of the cylinder and but very weakly and most rarely present in the cells of the gastro-vascular cavity. And that once the animal is complete and functioning sulfhydryl is only found where digestion of ingested food is taking place; is not found when the animals are starving.

These sequences of shift in localization and intensity of sulfhydryl concentrations running parallel as they do in orderly progression with what may be presumed to be the usual sequence in localization of proliferation intensity consequent upon the development of an *Obelia* hydranth and stolon, may be taken, in view of all that has gone before, as further substantiation in extension to another form of Hammett's postulate of a specificity of relation between sulfhydryl and cell increase in number. It also gives chemical demonstration of the validity of Hammett's growth analysis of *Obelia* hydranths ('35a).

That this identifies the growing endoderm as probably the primary proliferating tissue of *Obelia* does not deny proliferation properties to the ectoderm. Cell increase in number obviously takes place in this layer as in the endoderm. The fact that sulfhydryl is occasionally found therein is consistent with this. But the fact that disulfide is usual in ectoderm while sulfhydryl is usual in endoderm indicates or at least suggests that proliferation activity in ectoderm is secondary to that of endoderm. Whether or not disulfide is also present in actively proliferating endoderm cannot be determined by the nitroprusside reaction in this instance (Hammett and Chapman '37).

SUMMARY AND CONCLUSIONS

Procedure is described for the demonstration of sulfhydryl and disulfide in *Obelia geniculata*.

There is definite localization of free —SH in all regions of active proliferative growth. This is concentrated in the endoderm.

The mouth and stomach cavities of *Obelia* show a diffuse reaction for both chemical groups. This is evidence of hydranth digestive and transportational activity.

Regressing and senile animals give a diffuse test for sulfhydryl and disulfide within the core of the diminishing mass. This is evidence of endogenous catabolism.

The conclusion from these *in vivo* studies of chemical reactions under the microscope is that the observations agree with the concept of a specificity of relation between sulfhydryl and cell increase in number and suggests the endoderm is the primary proliferating layer of *Obelia*. They also allow the conclusion that *Obelia geniculata* ingests its food and carries on its intermediary metabolism quite like higher forms in principle.

The author wishes to acknowledge the guidance of Dr. Frederick S. Hammett throughout this work.

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THE DIFFERENCE IN THE WEIGHT OF THE LEFT AND RIGHT KIDNEYS

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Arataki ('26) weighed the kidneys of 34 albino rats between birth and 350 days of age and the averages of the groups showed the right kidney to be 2.1 per cent heavier than the left in the male and 2.3 per cent heavier in the female. The differences are slight but since the right kidney was also the heavier in most of the individuals comprising the groups he concluded that they appeared to represent a real, though small, inequality. The accumulation of kidney weight data for 548 male and 382 female rats has presented the opportunity for comparing the weight of the two kidneys in more detail. The method of removing and weighing the kidneys has been described (MacKay and MacKay '27). Arataki apparently did not decapsulate the kidneys before they were weighed. Our kidneys were decapsulated and an attempt was made to remove the excess of blood and urine which they contain.

The average weight of the two kidneys for various age-sex groups is presented in Table 1. There is no evidence of any age differences. In only two of the 58 groups were a majority of the left kidneys larger than the right. In all the rest the right organ was heavier. On the average the right kidney is 3.0 per cent heavier than the left in the male and 3.9 per cent heavier in the female. The right kidney was heavier in 75 per cent of the males and 76 per cent of the females. We agree with Arataki that the right kidney is usually the heavier and that the difference is significant.

Of the various factors which influence the weight of the kidney the amount of protein in the diet is one of the most important (MacKay and MacKay '31). Data were available to examine the influence of changes in total kidney weight produced by the dietary protein upon the weight relationship of the two kidneys. The diet protein was casein. The diets were fed for from 44 to 54 days before death. There is no evidence (Table 2) that the fluctuation in kidney weight produced by changing the protein intake has any influences upon the relative weight of the two kidneys.

The right kidney is heavier than the left kidney in both sexes. The average difference is slightly greater in the females than in the males but this sex difference is not statistically significant. Nor is the difference in the weight of the two kidneys related to age. It is, apparently, due to the operation of some prenatal factor. It is possible that a difference in the blood supply of the two organs may cause the weight difference. The right renal artery usually if not always leaves the aorta before the left one and is noticeably shorter. If the branching angles of the renal vessels are about the same this would tend to give the right kidney a greater blood supply. Drury ('32) has made a nice demonstration of the relation of the size of the kidney to its blood supply.

SUMMARY

In 548 male and 382 female albino rats between 35 and 760 days of age the right kidney is 3.0 per cent heavier than the left in the male and 3.9 per cent heavier in the female on the average. The right kidney was heavier in 75 per cent of the males and 76 per cent of the females.

Changes in renal weight produced by variation in the dietary protein intake had no influence on the relationship of the weight of the two kidneys.

It is suggested that the shorter right renal artery leaving the aorta nearer

TABLE 1

	MALE RATS					FEMALE RATS				
Age	No. rats in group	Mean kidney weight		Excess weight rt. kidney as as % of left kidney	Percentage of group in which right kidney was heavier	No. rats in group	Mean kidney weight		Excess weight rt. kidney as % of left kidney	Percentage of group in which right kidney was heavier
		left	right				left	right		
		mgm.	mgm.				mgm.	mgm.		
35	18	306	319	4.3	89	16	294	307	4.4	100
40	19	424	441	4.0	74	18	338	352	4.1	83
45	18	451	469	4.0	78	18	405	387		16
50	17	491	494	0.6	59	18	426	445	4.0	83
60	17	588	614	4.4	71	18	490	516	5.3	83
70	25	638	652	2.2	76	25	441	457	3.6	76
80	17	796	816	2.5	71	18	526	552	4.9	89
90	18	772	806	4.4	78	18	563	587	4.3	89
95	12	742	762	2.7	58	12	548	575	4.9	83
100	12	849	851	0.2	75	12	565	587	3.9	67
105	12	798	826	3.5	83	12	561	585	4.3	67
110	12	832	852	2.4	67	12	516	541	4.8	100
120	28	854	891	4.3	82	12	540	561	3.9	83
130	25	839	862	2.7	72	28	506	517	2.2	67
140	12	905	924	2.1	75	12	541	552	2.0	67
150	13	956	976	2.1	77					
180	12	1006	1046	4.0	75	11	610	645	5.7	91
185	12	843	876	3.9	67	11	556	565	1.8	73
190	12	868	906	4.4	92	11	521	559	7.3	91
195	12	840	866	3.1	75	21	586	606	3.4	67
200	11	920	946	2.8	64	13	578	607	5.0	92
210	11	881	914	3.8	73	11	576	586	1.7	64
220	24	977	1003	2.7	75	19	594	622	4.7	79
230	12	921	949	3.0	75	12	581	599	3.1	83
310	27	969	989	2.1	74					
365	13	972	1003	3.2	100					
370	12	986	1000	1.4	42					
375	12	935	944	1.0	58					
380	12	1001	1003	0.2	58					
390	12	991	1020	2.9	67					
400	22	1016	1051	3.5	82	24	622	658	5.8	83
410	12	980	1019	4.0	75					
580	23	1071	1123	4.9	87					
760	22	1005	1045	4.0	91					

TABLE 2
MALE RATS

Protein in diet per cent	No. rats in group	Mean kidney weight		Excess weight rt. kidney as % of left kidney	Percentage of group in which rt. kidney was heavier
		Left	Right		
		mgm.	mgm.		
		70 days old			
12	18	627	654	4.3	83
18	25	638	652	2.2	76
25	22	717	740	3.2	77
31	18	827	843	1.9	83
43	21	816	846	3.7	91
55	22	886	911	2.8	77
67	29	944	986	4.5	83
		130 days old			
18	25	839	862	2.7	72
43	15	1001	1009	0.8	67
67	15	1009	1030	2.1	63
		220 days old			
18	24	977	1003	2.7	75
43	16	1100	1110	0.9	56
67	16	1190	1220	2.5	75
		400 days old			
12	14	1016	1033	1.7	57
18	22	1016	1051	3.5	82
31	13	1102	1103	0.1	54
67	26	1136	1162	2.3	77

the heart than the left one gives the right kidney a greater blood supply and tends to make this organ the larger.

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DIFFUSION AND BIOLOGICAL MEMBRANE PERMEABILITY. I.

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An outstanding biological phenomenon is that of material transport through cell membranes and within cells, which is an intrinsic aspect of cell metabolism. The theoretical investigations of N. Rashevsky, ('33; '34 a, b, c; '35 a, b, c; '36), as well as a host of experimental correlations, suggest that the studies of cell permeability should have a special relevance in the study of the growth of cells and cellular aggregates. Permeability changes are known to be associated with mitosis, with the fertilization of eggs, and with the characteristic differences between various types of tissue, such as nerve cells as against other cells of the organism, or tumor against normal cells. It is one of the chief aims of the theory eventually to account in a quantitative way for as many of these phenomena as possible, in terms of the postulated physico-chemical mechanism. The consideration of such problems, and their complete solution, involves not only the physical constants of the cell, but also those constants which are partly characteristic of cell structure and partly of the specific state of diffusion. A definite specification of cell behavior insofar as it depends upon the metabolic activity of the cell requires, therefore, a comprehensive *physical* theory of diffusion constants; for these may vary in many ways, and by the manner and direction of their variation alter the functional and histological characteristics of the cell. In this paper, the first of a series, such a theory will be developed in approximate form. It will be a kinetic-molecular theory of the cell and the membrane viewed as liquid or gel, and will give the diffusion constants as functions of the cell constants and of the diffusing substance in a given state of diffusion.

I

We consider a spherical cell, consisting of two phases: the cytoplasm or internal phase, spherical in shape, and the membrane, a spherical shell adjoining the cytoplasm. For sake of simplicity the complications which might be introduced by a nucleus are here neglected, an approximation whose justification has been considered by N. Rashevsky ('34a). The external medium extends from the exterior boundary of the membrane out practically to infinity, that is, far enough from the cell so that at such a distance the presence of the cell practically no longer modifies the medium at all.

The two phases of the cell are colloidal sols or gels. At first, for convenience, we shall assume them to be homogeneous in chemical and physical composition; i.e., they may be mixtures of several types of colloid and crystalloid, but their composition and state of aggregation do not vary from point to point. The diffusing substance is held in solution in them, the solution being for our purposes considered sufficiently dilute to be treated as an ideal solution. No chem-

ical reactions occur in the membrane or in the external medium, but the cytoplasm is the seat of some type of metabolic activity, which for the time is left unspecified. In consequence of this metabolism, the concentration of the diffusing substance varies from point to point in all three phases, being in each one a continuous function of position, with possible discontinuities at the phase boundaries. Because of the spherical symmetry of the system, the concentration varies only in a radial direction, and we characterize its variation in terms of the radial distance from the centre of the cell, which is thus the origin of a system of spherical coordinates.

Since the membrane will be the focus of attention in this study, we give at this point a more particular description of its characteristics in our model. It consists of one or more types of spherical molecules of radial dimensions about 10^{-8} cm., which may represent water, or any other ordinary liquid, or any crystalloid in solution in same, together with one or more sets of larger particles, representing the colloidal macromolecules or micellae. They are to be generally of an elongated shape, approximated geometrically by prolate ellipsoids of revolution, whose long axes shall be of the order of 10^{-6} cm. in length, or greater. We shall for the most part neglect the thermal motions of all the particles described, with two exceptions which we shall note later. About the distribution of the orientations of the ellipsoids a number of hypotheses may be made, of which only one of the simpler ones is to be considered here.

The fundamental equation of the theory is Fick's Law, which gives the connection between the manner in which the concentration varies from point to point and the diffusion current or diffusion flow, J, which is defined as the quantity of the diffusing substance in grams crossing unit area of any surface in unit time. If any forces besides those due to the gradient of concentration act upon the diffusing particles, this equation takes the form

$$J = -D \operatorname{grad} c + Bfc \tag{1}$$

where D is the coefficient of diffusion of the substance in the particular medium, f is the force acting on a single diffusing particle, c is the concentration of the diffusing substance in g. cm^{-3}, and B is the mobility of the diffusing substance, which is connected with D by the relation (Herzfeld, '26)

$$B = D/kT. \tag{2}$$

Furthermore, the force f acting on a particle (which we attribute to the molecular forces of all the constituents of the medium), is by our hypothesis of spherical symmetry a function only of the radial distance r from the centre of the cell, as is c, and grad c has the form dc/dr.

Equ. (1) constitutes a differential equation for the determination of c, if J were known. We treat J as a known function of r, and proceed to integrate the equation by the classical methods (Lense and Radacovic, '28). If we do so, with the condition that c assumes the values c_1 and c_2 at the values of r equal to r_1 and r_2 respectively, we obtain the relationship:

$$c_1 e^{-U_1} - c_2 e^{-U_2} = \int_{r_1}^{r_2} (J/D) e^{-U} dr, \tag{3}$$

where

$$U = \int_{\infty}^{r} (f/kT) dr \tag{4}$$

while U_1 and U_2 are obtained from U by substituting r_1 and r_2 respectively in the upper limit of the integral. U is clearly identical, except for the factor $-1/kT$, with the potential energy of a diffusing particle at the point or level r.

We interpret the boundary condition which we have used as follows: c_1 and c_2 are the values taken on by c just inside and just outside the membrane respectively, and r_1 and r_2 are thus the interior and exterior radii of the membrane, so that their difference $r_2 - r_1$ is the thickness of the membrane, which we denote by δ.

By introducing an approximation at this point it is now possible to invert (3) to obtain a value for the diffusion current across the membrane, that is, the average value of J in the membrane phase. In a spherical system J is not a constant; for, since it represents the flow across unit area, and the area of a sphere of radius r varies like r^2, J varies as $1/r^2$. If the curvature of the surface were absent J would be strictly a constant. But our model of the membrane implies that its thickness is very small compared to the dimensions of the cell, so that J varies by a negligible amount as we pass across from r_1 to r_2. Thus it can be treated as practically constant in this interval, and removed from under the integral sign in (3). Also, since the solution has been supposed dilute, D is constant in the membrane by a similar consideration, and may also be taken out of the integral, while we denote the value which it has in this interval by D_m. The expression for the flow across the membrane per unit area and unit time in the direction of increasing r now takes the form:

$$J = (c_1 e^{-U_1} - c_2 e^{-U_2}) D_m / \int_{r_1}^{r_2} e^{-U} dr. \tag{5}$$

The integral in the denominator can, of course, not be evaluated until some more definite hypothesis has been made concerning the nature of the intermolecular forces, and the same holds true for the form of U_1 and U_2. Thus the exponential factors in (5) present a certain difficulty for the theory in its present form. We know the exponents to represent the potential energies of a diffusing particle at the given points, divided by kT, which is practically the average kinetic energy of the particle. From the hypothesis of distinct phases, it is reasonable to conjecture that U_1 and U_2 will be representable as a sum of terms pertaining only to the membrane phase and terms due to the influence of the appropriate adjacent phase. If we suppose that the force field of the membrane particles is the field of electric dipoles or multipoles, the potential will clearly involve the concentration and the structure of the membrane particles in some as yet unknown way; we can tell only that it will be proportional to the con-

centration n_m and to the dipole moment, the latter being plausibly (Smyth, '31) taken proportional to the linear dimension of the particle. But what other involvements of the constants of the particles may exist, and whether the various terms of U are positive or negative, cannot be told until a specific hypothesis concerning the forces is taken and worked out in detail. It is this which we propose to do in a paper which will shortly follow this one. In the meantime, the analysis of the results of the theory in the present stage may best be carried out for a special case, that in which all potentials are zero or practically so (at least very much smaller than kT, which is of the order of 10^{-14} ergs deg.$^{-1}$). In this case all the exponential terms become equal to unity, and the integral in the denominator of (5) becomes $r_2 - r_1 = \delta$.

But before proceeding in this way it may be interesting to rearrange slightly the expression (5) so as to give a comparison with previous empirical formulae for J. The classical expression for the flow across a membrane is:

$$J = h(c_1 - c_2), \tag{6}$$

which was corrected by Rashevsky to

$$J = h(\alpha c_1 - c_2), \tag{7}$$

α being the partition coefficient of the diffusing substance between the cytoplasm and the external medium; that is, the ratio of the value which the cytoplasm concentration takes on at r_1 to the value which the external concentration takes on at r_2, when the diffusing substance is in statistical equilibrium under the influence of the field of force only in the absence of any gradients of concentration (Fowler, '29). But we can rearrange (5) by factoring out e^{-U_2}; expressing the denominator of (5) simply as a constant $1/A$:

$$J = Ae^{-U_2}D_m(e^{-(U_1-U_2)}c_1 - c_2).$$

But the exponential coefficient of c_1 is exactly the definition of the partition coefficient α, so that (5) is the same as (7) if we set:

$$h = Ae^{-U_2}D_m. \tag{8}$$

This proportionality factor h is what is usually called the coefficient of permeability of the membrane, and has the dimensions of cm. sec.$^{-1}$. It is, as may be seen from the equations, a measure of the penetrability of the membrane; but, since U_1 and U_2 contain contributions from the membrane also, it is not a unique measure even for a given c_1 and c_2—the partition coefficient must also be known to give the flow J. It is for this reason that in this paper we have analyzed J directly, rather than attempting immediately to proceed in terms of h.

In the special case of zero potentials which we are to consider next, the exponentials all become unity, and $A = 1/\delta$. We are thus reduced to the expression (6), with:

$$h = D_m/\delta. \tag{9}$$

Here h is a unique measure of the flow for a given $(c_1-c_2)_2$ and the further analysis may for convenience be referred to (9). In all that follows it must be remembered that, when the potentials are introduced, they may modify or even reverse the results, since for sufficiently high exponent an exponential expression will usually outweigh even the highest degree algebraic expression. Our findings will perhaps apply to many types of experimental data, but in all likelihood apply with most certainty to plant cell membranes and others of comparatively inert type.

II

The next step indicated is a closer scrutiny of the diffusion coefficient. The first approach which suggests itself is to use the Einstein formula (Stiles, '24a) for D_m:

$$D_m = kT/6\pi\eta_m\rho \tag{10}$$

where η_m is the viscosity of the membrane, and ρ the radius of the solute molecule (represented by a sphere). There are, however, logical difficulties in the hydrodynamical foundation of (10), and in view of that it seems more desirable to employ a consistent kinetic-molecular approach to the problem.

The treatment of the kinetic theory of gases comes immediately to hand for this purpose. The diffusion coefficient for the solute is given (Jeans, '16a) as:

$$D_m = \lambda\bar{c}/3 \tag{11}$$

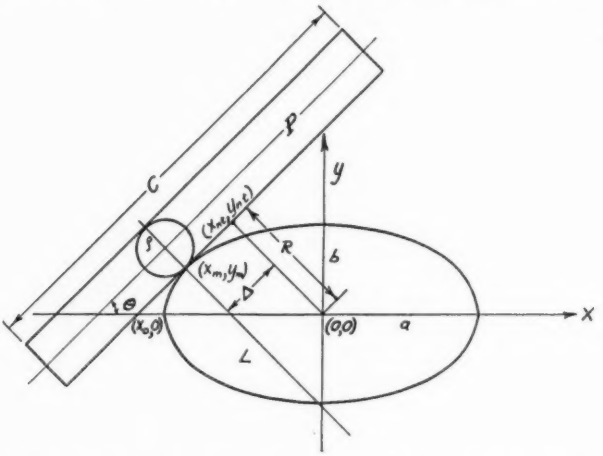
where λ is the mean free path of the solute or diffusing particle in the medium, and $\bar{c}$ is its mean velocity. This formula implies that the molecules of the medium are not in motion, and this approximation, to which we referred earlier, is taken for the sake of simplicity. It probably holds better for the colloidal micellae of the medium than for any of its other constituents, for the huge micellae may be expected to have a rather slow thermal motion.

The calculation of the mean free path involves consideration of collision probabilities between the spherical molecules of the solute and the ellipsoidal molecules of the colloid constituent of the membrane, as well as the ordinary type of collision between two spheres for the solute and the crystalloidal or aqueous constituents. The calculations follow, with some necessary modifications, the classical method (Jeans, '16b). They are not altogether rigorous, for they also omit the fact that there is a three-dimensional distribution of the directions of motion of the diffusing molecules, that they are not all moving either forward or backward in one direction. The various omissions have been made in order to avoid the mathematical complications of the more exact computation, and it seems safe to assert that the results are correct well within the limit of experimental error in biological observations of the diffusion process in the cell.

The mean free path according to Maxwell (Jeans, '16c), is given by:

$$\lambda = 1/[\pi\textstyle\sum_j n_j S_j^2(1 + m_d/m_j)^{1/2}] \tag{12}$$

where the summation is taken over all the types of particle with which the diffusing particle may collide, including those of its own kind, the n_j are molecular densities expressed in number of molecules per cm.^{3}, the m_d are the molecular masses in grams, and the S_j for each type of collision is the radius of a cylinder whose axis is formed by the rectilinear path of the diffusing molecule, and such that, if the centre of the other particle lies within the cylinder, a collision will occur. S_j is calculated as follows: The diffusing particle, a sphere of radius ρ_d, sweeps out a cylinder of the same radius. Let the other type of particle be represented by an ellipsoid, and let its orientation relative to the motion of the sphere be given by two angles θ and ϕ. Take an ellipsoid of a given orientation, so placed as to have a common tangent plane with the cylinder swept out by the sphere. The perpendicular distance from the centre of the ellipsoid to the axis of the cylinder is the value of S_j for the given orientation. The value of S_j^2 to be used in (12) is obtained by multiplying S_j^2 (θ, ϕ) by the distribution of



orientations, that is, the probability of a given pair θ and ϕ, and averaging over all orientations. When the other particle is also a sphere, S_j is simply the sum of the radius ρ_d and the radius of the other sphere, say ρ_s. For an ellipsoid, because of the rotational symmetry, it is only necessary to specify the orientation by the angle formed by the path of the particle and the projection of the major axis of the ellipsoid on a plane containing the path (θ), and the angle between the major axis and the given plane (ϕ). The distribution function of orientations may be chosen in a number of ways. In this paper a random orientation will be studied.

In the diagram p is the path of the particle of radius ρ_d and lies in the plane of the paper, which is taken as the reference plane. The ellipse, having major and minor semi-axes of length a and b respectively, is the principal meridian curve or generating curve of the ellipsoid of revolution; its equation referred to its own principal axes is

(13) $$x^2/a^2 + y^2/b^2 = 1.$$

If $m = \tan\theta$ is the slope of the ellipse at the point of tangency, the coordinates of that point are given by x_m and y_m:

(14) $$x_m^2/a^2 = m^2/(m^2 + b^2/a^2); \qquad y_m^2/b^2 = (b/a)^2/(m^2 + b^2/a^2),$$

and from the equation of the tangent line the coordinate of its intercept on the x-axis is:

(15) $$x_0 = x_m - y_m/m$$

where the signs are chosen, according to the construction, as follows:

(16) $$x_m = -(x_m^2)^{1/2}; \qquad y_m = (y_m^2)^{1/2}.$$

The coordinates of the intersection of the tangent line and the perpendicular upon it from the origin are:

(17) $$x_{nt} = m(mx_m - y_m)/(1 + m^2); \qquad y_{nt} = -(mx_m - y_m)/(1 + m^2).$$

Furthermore

(18) $$\Delta^2 = (x_{nt} - x_m)^2 + (y_{nt} - y_m)^2 = (my_m + x_m)^2$$

(19) $$R^2 = x_0^2 \sin^2\theta = (y_m - mx_m)^2/(1 + m^2).$$

If now the ellipse is rotated out of the plane through an angle ϕ, the axis of rotation being L, the diameter of the sphere ρ_d produced, it constitutes the meridian curve of the ellipsoid in the orientation θ, ϕ, and construction gives S^2 as:

(20) $$S^2 = (\rho_d + R)^2 + \Delta^2 \sin^2\phi.$$

Now, by (14), (18), and (19), and the definition of m:

(21) $$\Delta^2 = (a^2 - b^2)^2 \sin^2\theta/(a^2 \sin^2\theta + b^2 \cos^2\theta)$$

(22) $$R^2 = a^2 \sin^2\theta + b^2 \cos^2\theta.$$

The average value of S^2 with the chosen random distribution of orientations is then

(23) $$S_m^2 = \int_0^{2\pi}\int_0^{\pi} S^2(\theta, \phi) \sin\theta d\theta d\phi \Big/ \int_0^{2\pi}\int_0^{\pi} \sin\theta d\theta d\phi.$$

Carrying out the integrations, this becomes:

(24) $$S_m^2 = (\rho_d^2 + a^2) + \tfrac{1}{2}(a^2 - b^2)\{\tfrac{1}{3} - \tfrac{1}{2}(b^2/a^2)(1/E) \ln[(1 + E)/(1 - E)]\} + \rho_d(b + a(\sin^{-1}E)/E)$$

where $E = (1 - b^2/a^2)^{1/2}$ is the eccentricity of the ellipsoid. For $b = a$ this reduces, as it should, to $(\rho_d + a)^2$, the value appropriate to the collision of two spheres. Now, as b increases toward a, the second term varies from a^2 to 0, while $(\sin^{-1}E)/E$ varies from $\frac{1}{2}\pi$ to unity. Thus for the normal membrane, where the relative or-

ders of magnitude of ρ_d and a are as given previously, practically all terms except a^2 in the first bracket may be neglected. But since we will wish to consider variations of the state of aggregation which involve reducing considerably the value of a, we will wish to refer to (24) in full. We will introduce S_m^2 into (12) as a symbol given by (24), remembering that for the normal membrane, when we are not considering changes of state, particularly peptization, it is practically equal to a^2, and returning to (24) for a less approximate analysis when necessary.

It is also to be noted that for most situations to be considered the factor $(1+m_d/m_j)^{1/2}$, when j refers to the colloid particles, is practically equal to unity due to the high molecular masses of the colloid molecules.

At this point it is necessary to introduce a correction into the classical calculation of λ (Jäger, '19). This is a volume factor of the Van der Waals' type, due particularly to the large molecular volume of the colloid particles, but taking into account also the spherical molecules of the phase, and even the molecules of the diffusing substance itself. This consists simply in multiplying λ by $(V-b)/V$, where V is the total volume of the phase, and b is the Van der Waals' effective volume occupied by the molecules in V. This correction may be written as $1-b/V=1-\omega$, and can be expressed additively by the contributions from the different molecular species. Indicating the terms by the same indices as we have used previously, we have

$$\omega_d \cong 4n_d v_d \tag{25}$$

$$\omega_s = 4\pi(\rho_d + \rho_s)^3 n_s/3 \cong 8n_s v_s \tag{26}$$

$$\omega_m = n_m v_m (a/b). \tag{27}$$

The first of these is approximate only, since n_α is a function of position; but, as it varies only along the radial direction, its variation within the membrane may to a good approximation be neglected. It represents in general, for a dilute solution, a very small contribution to ω. The approximate alternative expression in (26) holds if ρ_d and ρ_s are of the same order of magnitude; if ρ_s is much larger, it has a factor 1 instead of 8. (27) is obtained by a modification of Staudinger's expression (Staudinger, '32) for the effective volume excluded by colloidal particles of an ellipsoidal or rodlike shape, which he takes to be the volume of a cylinder having a diameter equal to the major axis, and height equal to the minor axis of the ellipsoid or rod; we take the corresponding oblate ellipsoid of revolution instead of a cylinder. This formulation implies a rotational motion of the ellipsoid, and is probably not exact, since it assumes free rotation, and assumes that the volume excluded is equal to the entire volume swept out by the rotation, whereas it is probably only some fraction of same. But the order of magnitude, and the involvement of the factor a/b, are probably correct. v_j is the calculated molecular volume of one molecule of species j.

The expression for D_m, neglecting the molecular mass of the diffusing substance in comparison with that of the colloid particles, may be written:

$$D_m = \bar{c}(1-\omega)/3\pi\left[n_m S_m^2 + n_s(\rho_d + \rho_s)^2(1 + m_d/m_s)^{1/2} + 4(2)^{1/2} n_d \rho_d^2\right], \tag{28}$$

ω being given by the sum of (25), (26), and (27), while S_m^2 is given by (24). The diffusion current J is directly proportional to this expression, as we see from (5).

III

We are now in a position to examine the results of the preceding analysis, and to consider how the parameters of the membrane and of the diffusing substance, as well as the parameters of changes of the state of aggregation of the membrane, effect the diffusion process. It will be noted that the above calculations have been carried out for the case of only one kind of spherical particle and one kind of ellipsoidal particle constituting the membrane phase. But since the denominator of λ, and hence of D_m and J, is a sum of terms, it is evident that our results are easily generalized to the case of any number of constituents.

At this point it will be useful to consider some of the orders of magnitude involved among our physical constants. ρ_d has already been specified as 10^{-8} cm., while ρ_s, a, and b vary from that value to 10^{-5} cm., d being usually much larger than the others. The molecular masses of the non-colloids, such as m_d, lie in the neighborhood of 10^{-20} gram or less. The external radius of the cell, r^2, is about 10^{-3} cm., while $r_2 - r_1 = \delta$, the thickness of the membrane, may vary from as small as 10^{-6} cm. to as much as 10^{-4} cm.; unless the colloid particles are all aligned tangentially, it must be at least as great as the value chosen for $2a$, since the membrane cannot be less than a monomolecular layer. n_s, for densities not differing greatly from that of water, and the given order of magnitude for m_s, is about 10^{23} cm.$^{-3}$, while n_d is between 10^{16} and 10^{18} cm.$^{-3}$ (estimated from the concentrations given by N. Rashevsky ('35a), using the relation $n = Nc/M$, where N is Avogadro's number and M the molecular weight). The thermal velocity $\bar{c}$ of the diffusing molecules in the membrane is probably between 10^3 and 10^4 cm. sec.$^{-1}$. From the figures of N. Rashevsky, we may estimate the diffusion coefficient to be of the order of 10^{-7} cm.^{2} sec.$^{-1}$ or higher, perhaps as high as 10^{-5} cm.^{2} sec.$^{-1}$ in some cases. The colloid concentrations probably lie around the other figures; i.e., n_m about 10^{18} to 10^{22} cm.$^{-3}$.

From the estimate of Rashevsky for the value of D_m, and the other orders of magnitude, it may be seen by simple arithmetic that a set of values may be chosen from the range permissible for the various constants, consistent with one another and giving the right order of magnitude. An additional restriction is placed upon these values, however, which narrows them down even more; for it is necessary for $(1-\omega)$ to be positive, both because an effective volume greater than the actual volume in which the molecules lie would be a physical absurdity, and because a negative diffusion coefficient is meaningless. In special cases the values of the constants may thus be narrowed down considerably. Suppose for example that $\bar{c} = 10^3$ cm. sec.$^{-1}$, and that D_m is between 10^{-6} and 10^{-7} cm.^{2} sec.$^{-1}$. The inequalities which we use take the approximate form

$$1 > (1 - \omega) \geqq \Omega_0 \tag{29}$$

the latter figure being chosen arbitrarily as a lower limit since it is unusual even in solids for the closest packing to give a zero free volume that is, $(1 - \Omega_0)/4\pi b$

(30) $$n_m a^2 < 1/\pi b$$

(31) $$(\tfrac{1}{4})(\bar{c}/3\pi n_m a^2) \leqq 10^{-6}.$$

Because of (30) b must be less than 10^{-8} cm. Taking $b = .75 \times 10^{-8}$, and combining the three inequalities, we obtain

(32) $$\tfrac{1}{5} \times 10^8 \leqq n_m a^2 < \tfrac{1}{4} \times 10^8$$

from which it is evident that only certain pairs of values of n_m and a are permissible, as, approximately 10^{17} and 10^{-5}, 10^{19} and 10^{-6}, 10^{21} and 10^{-7}. This calculation is rough and approximate, especially since it has been carried through only for one species of membrane particle; but it serves to indicate how, with more information and more accurate data, the theory may serve to give some precise knowledge of the constitution of the membrane (and, for that matter, of the cytoplasm, since for it a similar diffusion coefficient exists).

The permeability is first of all inversely proportional to the thickness of the membrane. This is what we should expect within the permissible variation of that quantity.

The diffusion coefficient is proportional to $\bar{c}$, which is proportional to $T^{1/2}$, T being the absolute temperature. That is, other things being equal, permeability should increase with temperature, which agrees with the experimental findings (Stiles, '24b). We note also that the absence of what Osterhout (Gellhorn, '29a) calls a *unique temperature quotient* of permeability is explicable upon ground of the potential terms which we have dropped, for these are proportional to $1/kT$, and probably influence the permeability more in many cases than the factor $\bar{c}$.

$\bar{c}$ is likewise proportional to $m_d^{-1/2}$, while the denominator of D_m also increases with m_d (see (28)). That is, for given molecular volume of the diffusing substance, and a fixed membrane type, the permeability decreases with the molecular mass. This is in agreement with the experimental findings (Gellhorn, '29b), which indicate, superimposed upon the valence effect which J. Loeb found for ion series of the type encountered in permeability observations as well as for other colloidal phenomena, an effect of molecular mass.

Again, the factor $(1-\omega)$ decreases with the molecular volume or molecular radius of the diffusing substance, while the denominator increases with the molecular radius. Thus we have the familiar decrease of permeability with increase of molecular volume of the penetrating substance which has been noted by many investigators, and which the ultra-filter or molecular filter theory of permeability (Gellhorn, '29c) was devised to explain.

The permeability is seen in the same way to decrease with increase of the density and molecular volume of the membrane constituents, and rather strongly with the increase of the long axis of the colloid particles, which is a physically natural result, consistent with those of the preceding paragraph, for such a variation gives, in terms of the sieve theory, a decrease of pore size. It is worthy of note that, if the substance indicated by the index s is not a membrane constituent, but another diffusing substance, agreement with the data is again

found (Gellhorn, '29d); the permeability of the membrane for one substance is lowered by another substance which also penetrates according to the molecular volume and mass of the second substance (again neglecting valence effects).

We come finally to what is probably the most interesting result of the theory. Besides changes in the characteristics of the diffusing substance and the density of the membrane, we may change the state of aggregation of the colloidal membrane constituents, leaving fixed the total quantity present. Retaining our approximate representation of the colloid molecules as ellipsoids, we may divide such changes into two types: those which increase the length and those which increase the thickness of the ellipsoids. Let us lump together all terms not depending on a and b, since they will simply be additive constants in numerator and denominator. For this type of change $n_m v_m$, the relative volume occupied by the colloid (neglecting swelling, etc.), is to be constant, so that we may write $n_m$where it appears in the denominator as ϕ_0/ab^2. The constant ϕ_0 is the fixed value of $n_m v_m$ times $3/4\pi$. From (9) and (28) h may be written

$$h = \bar{c}(A - n_m v_m a/b)/(\phi_0 S_m^2/ab^2 + B)3\pi\delta$$

or

$$h = \bar{c}(A - n_m v_m a/b)/\{\phi_0[\rho_d^2/ab^2 + a/b^2 + a/6b^2 - \epsilon_1/4a - 1/6a + \epsilon_1 b^2/4a^3 + \rho_d/ab + \rho_d\epsilon_2/b^2] + B\}3\pi\delta \tag{33}$$

where $A = 1 - 4n_d v_\alpha - 4\pi(\rho_d + \rho_e)^3 n_s/3$ is a constant less than unity, and probably greater than $3/4$, while B is a constant of the order of 10^7. In this form we have a measure of the variation of h with the change of state of aggregation of the colloids of the membrane. B is by simple algebra equal to $\sum_j n_j S_j^2$, where j ranges over all indices except m, the index referring to the ellipsoids.

A further simplification is introduced by noting that in S_m^2 the factors involving E vary hardly at all over the range of a and b. We have in fact as b goes from 6 min to a:

$$15 \geqq E^{-1} \ln [(1 + E)/(1 - E)] \geqq 2 \tag{34}$$

$$(\tfrac{1}{2})\pi \geqq (\sin^{-1} E)/E \geqq 1. \tag{35}$$

It is therefore permissible to regard these expressions as coefficients, constant and of the order of unity, in S_m^2. We call them ϵ_1 and ϵ_2 respectively.

It may also seem at first as if the form of E imposed a further restriction, beyond (29)–(32), on the variation of a and b, so as to keep all expressions real. But we can easily show that this is not so; for, if we expand (Duschek, '28) the logarithm and $\sin^{-1}$, they are found to be expressible in series containing only odd powers of E, and no constant term. Dividing them by E, the resulting series contain only terms of even degree, and are therefore always real.

A further restriction is placed, however, by (29), which is to hold for all a and b. Suppose for some initial set a, b, (29) is satisfied; that is, $n_m v_m a/b \leqq A \leqq 1$. Then if a resp. b is changed:

$$a \leqq Ab/n_m v_m \tag{36}$$

$$b \geqq a n_m v_m / A . \tag{37}$$

The second of these is always true if (29) is satisfied; the first sets an upper bound to the increase of a (or *decrease* of b); for if initially the ellipsoids are such as to occupy a certain fraction of the total phase volume, the variation of their shape cannot cause this fraction to exceed the total phase volume.

We consider first the variation of a keeping b fixed. Suppose the total relative volume $n_m v_m$ is about 10^{-2}, and b about 10^{-8} cm. Then by (36) a cannot exceed 10^{-6} cm. If the relative volume is 10^{-4}, a can go as high as 10^{-4} cm. The numerator of h decreases linearly with a rather sharp negative slope of 10^6 resp. 10^4. The denominator is a combination of linear and hyperbolic terms, and the constant B, the linear terms having a relatively small slope.

Now we consider the variation of a only from $a = b$ to $a = a^*$, the latter being the largest value which by the above conditions a may assume; similarly b varies only from its smallest plausible value, about 10^{-8} cm. by (37), to the value a. Thus in this instance, with fixed b, and neglecting the constant factor $3\pi\delta$, the denominator of h decreases slowly from a little over 10^7 to a shallow minimum for a about 10^{-4} cm., and then increases almost linearly. The variation of the denominator is kept sharply bounded by the factor ϕ_0 and the value of B; especially, the larger B, the more nearly constant is the denominator as a varies. The reciprocal of the denominator thus begins at about 10^{-7}, has a shallow maximum, and drops to about 10^{-8}. The variation of the numerator of h therefore dominates the entire phenomenon, and h in the case considered will decrease monotonically. Only if ϕ_0 is very high (and this is limited by the condition (29), or B very small (that is, the other constituents of the membrane present in small amounts) will the variation of the denominator be rather marked. But for large ϕ_0 the permissible variation of a will not reach the minimum value $a = 10^{-4}$ at all. Thus we see that only as the particular colloid considered becomes more and more nearly the total membrane constituent will the variation of h be other than monotonic; in such an event it may have an early maximum. Of course, it is to be remembered that a change of state in the membrane will affect all the components to some extent, so that this simple consideration based on the variation of one component alone should not be pushed too far.

Now consider the increase of b for fixed a equal to about 10^{-6} cm. The numerator of h increases from zero at b about 10^{-8} cm., and slowly arrives at its highest permissible value for $b = a$. The variation of the denominator is in this case practically monotonic, since the term in b^2 is very small compared with the others, and for large B the variation is again slow and hardly perceptible. For smaller B the variation would be sharply hyperbolic, the asymptotic value being practically attained for a tenfold increase of b. The reciprocal of the denominator thus shows for large B a small monotonic increase, and h again increases monotonically practically as its numerator. For small B the curve would still be monotonic, but would rise more steeply.

The results of this analysis, though of most interest in connection with the change of state of a given membrane, may give also a prediction of the comparative behavior of membranes of different *chemical* structure, depending on the shape of the molecules of the chief membrane constituents for a given concentration of the constituent, for shapes ranging from the tetrahedral or other high-symmetry configurations of the atoms to very long rods or chains. Moreover, interchanging the axes a and b we obtain a representation of molecules which have the shape of an *oblate* spheroid or disc, such as ring compounds.

For a discussion of change of state, it is necessary to ask what interpretation in the customary terminology of colloid chemistry is to be given to the variation of a and b. The following suggests itself as fairly plausible: An increase of a for fixed b implies the formation of chains of macromolecules, perhaps through secondary valence bonds or (as seems feasible in the case of proteins) through linkage of the carboxyl ion group of one zwitterion to the positive amino-hydrogen group of the next. This may simply result in an increased viscosity of the membrane, or it may even, the free rotation of the chains being hindered and orientation made easier, result in formation of a gel. To the decrease of a, on the contrary, corresponds not one picture, but two. For decrease from a high value of a may simply give a decrease of viscosity or a transformation from sol to gel, but after a certain point a could be decreased no further without the rupture of principal valence bonds and the hydrolysis of the basic macromolecules. In a similar way we consider the variation of b. For b increasing, we have a formation of bundles of parallel molecules, which may be interpreted as an association through side-chains or salt bridges, or as coagulation. The decrease of b may in a similar way represent a rupture of the associated molecules, or peptization of the coagulate.

This is somewhat speculative, however, owing to the neglect of the molecular forces, which are probably of most significance here. It is also quite difficult to get any sort of experimental check upon these points, upon the precise relation of permeability variations to the state of aggregation, save for somewhat rough correlations with swelling and viscosity changes, the former being suggested largely by comparison of the Hofmeister series (Gellhorn, '29e), for the two phenomena. Nowhere are the data as detailed as would be required to confirm or disconfirm the present theory; details as to the effect of external agencies on permeability are plentiful, but an exact connection between such agencies and the colloidal changes of state, expressed in terms of a definite molecular model, is lacking. What is required is in fact a mathematical theory of colloidal behavior, which in terms of a definite model will carry out quantitatively the considerations so ingeniously presented in qualitative form by Hamaker (Hamaker, '37). The detailed analysis of intermolecular forces which constitutes a part of the program of this investigation may constitute the first draft of such a theory.

In the meantime, the theory even for zero potential presents some points of interest. Apart from the interest presented by the possibilities if we vary several constituents at once, or consider splitting or forming of aggregates with unequal

parts, all of which we have neglected here, the trend of h with change of a resp. b seems in concordance with the fact that high-molecular compounds increase in viscosity, and in the complexity of their behavior in general, as the molecules become less symmetrical and more rodlike (assuming with regard to the first point that the viscosity of the membrane is correlated positively with difficulty of penetration, and noting with regard to the second the reversal of the direction of S_m^2/ab^2 for sufficiently high a).

The increase of permeability with b is more difficult to understand. We might suppose that perhaps a swelling of the colloid, which is conjectured to be correlated with rise of permeability, might give rise to an effect similar to the increase of b: but in such a case it would be hard to be sure that the relative volume, counting in the volume of bound water, would be constant. It might be so if swelling involved withdrawal of water from the external medium, in which case the membrane would increase in volume together with the volume of bound water. But if swelling meant simply an exchange of water between colloid and the rest of the membrane, the relative volume would increase, and if it increased fast enough the permeability, on our theory, might even decrease after reaching a maximum; although just possibly the change of relative volume might be merely enough to keep the numerator of h stationary while the denominator decreased monotonically.

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AN ESTIMATE OF THE CENTER OF THE RELIEF POPULATION OF THE UNITED STATES

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At the time of each decennial population census the Census Bureau reports the location of the center of population, which may be defined as the center of gravity[1] of the population. In more popular terms, it might be said to be the point which is most accessible to all the people in the United States, if they were to travel to it.

The theoretical method of obtaining the center of population is as follows: First, it is necessary to assume that the United States is a plane, rather than a curved surface. The center of population is the intersection of a line running from north to south, representing the average longitude of persons in the United States, and another running from east to west, representing the average latitude. In order to obtain the average latitude for the total population, the sum of the latitudes of all the people in the United States is divided by the total population. The same procedure is followed in order to obtain the average longitude.

The method used by the Bureau of the Census[2] differs from the theoretical method in important details. From a practical standpoint it is impossible to determine the latitude and longitude of every person in the United States. Therefore, the country is divided into small areas and all the people in an area are assumed to be clustered at one point. The Bureau of the Census divides the country into square degrees, areas bounded by adjacent meridians and parallels. These areas vary in size from 3,200 to 4,300 square miles because the size of a degree of longitude varies. The population of principal cities is excluded from these sections and taken account of separately. The center of each section is the geometric center unless there is an uneven distribution of population due to small cities or towns or geographic features, in which case the center of the section is determined by estimation. The average latitude and longitude of all persons is found by summing the products of the number of persons in each area multiplied by the latitude (or longitude as the case may be) of the center of the area and dividing by the total population. However, the arithmetical work can be materially reduced if the "short-cut method" of finding an arithmetic mean is used. In this method two arbitrary axes are chosen, one running north and south and the other east and west, in the neighborhood of where the center of population is expected to be. Distances are measured from these lines with regard to sign; that is, all distances north of the line running east and west are given a positive and those south a negative sign. Similarly, all distances east of a line running north and south are negative and distances west are positive. These relative distances are then multiplied by the number of persons in each

[1] The center of population is thus a function of distance as well as number of people and does not coincide with the median point of population, which ignores the factor of distance.

[2] Fifteenth Census of the United States, 1930, vol. 2, page 20.

square degree and each principal city and summed. The two sums (one for latitudes and one for longitudes), divided by the total population, yield the

FIG. 1

(⊡) denote centers of general and special relief population.
(+) denote centers of general relief population.
(⊙) denote centers of population, U. S. Census.
Numerals: 1, October 1933; 2, May 1934; 3, January 1935; 4, May 1935; 5, October 1935.

distances of the center of population from the two arbitrary axes. The centers of population from 1880 to 1930 are shown in Figure I and in Table I.

TABLE I

LOCATION OF CENTER OF POPULATION OF THE U. S. AND DISTANCE FROM 1930 CENTER BY DECADES, 1880 TO 1930

Year	Location of Center		Distance in Miles from 1930 Census		
	Latitude	Longitude	Northward	Eastward	Total Distance
1880	39° 4′ 8″	84°39′40″	0.5	133.1	133.1
1890	39°11′56″	85°32′53″	9.5	85.4	85.9
1900	39° 9′36″	85°48′54″	6.7	71.0	71.3
1910	39°10′12″	86°32′20″	7.4	32.1	32.9
1920	39°10′21″	86°43′15″	7.6	22.3	23.6
1930	39° 3′45″	87° 8′ 6″	—	—	—

A slightly different procedure had to be employed in order to determine the center of the relief population for October 1933. The only data available from the October 1933 Unemployment Relief Census that can be used in determining the center of the relief population are the number of persons on relief classified by counties, and by cities over 100,000 population in 1930.[3] The average size of a county is approximately 960 square miles, so that in finding the center of the relief population the country was divided into about four times as many areas as when the method of square degrees is employed. For cities of more than 100,000 population the persons on relief were assumed to be at the center of the city, and these persons were thenceforth excluded from the county in which the city was situated. The center of each county was assumed to be the geometric center except in cases where the population of the county did not seem to be evenly distributed, because of the presence of small cities, towns, or geographical features, in which case an adjustment was made in the estimated location of the center of the county. The maps used were from the Rand-McNally World Atlas (Premier Edition); the scales varied from eight to forty-five miles to the inch, most of them being between twenty and thirty-five miles to the inch. In finding the center of a county, the geometric center was estimated by inspection, which could be done within a small range of error. Then from the Population Census of 1930 it was noted whether the population of any towns or cities constituted twenty percent or more of the population of the entire county. If this were the case, the center was located between the geometric center and the city, the distance moved being proportional to the percentage of the population of the county that resided in the city. Thus, if 25 percent of the population of a given county resided in a city the center of the county was estimated to be one-quarter of the distance from the geometric center to the city. If there were no such city or town, the geometric center was assumed to be the population center of the county. It was found to be necessary to estimate the centers of about half of the counties by this procedure.

[3] Reports Number One and Two, Unemployment Relief Census, October 1933, Federal Emergency Relief Administration, Washington, 1934.

The distance in miles from the center of each county to the nearest latitude and longitude lines was then measured. This was also done for the cities of 100,000 or more population. In calculating the latitude and longitude of the center of population, the method of choosing two arbitrary axes from which to measure all centers was used. These two lines were 39° north latitude and 86° west longitude. The distance in miles from these axes was then obtained for each county. For example, if the center of the county were at 43°+10.8 miles north, 71°+9.3 miles west, then the distance from the latitude axis was +286.8 miles, since 43°+10.8 miles was 4°+10.8 miles from the latitude axis ($4\times69.0+10.8=286.8$ miles) and the distance from the longitude axis was −748.8 miles, since 71°+9.3 miles was −15°+9.3 miles from the longitude axis ($-15\times50.54+9.3=-748.8$ miles).[4]

After the distance of the center of each county and large city from the arbitrary axes had been obtained, the next step was to multiply each distance by the number of people on relief in that county or city. The sum of the products of latitude multiplied by the number of persons on relief, when divided by the total number of persons on relief, gave the distance in miles from the thirty-ninth degree of latitude to the average latitude of the relief population of October 1933. The same procedure was also employed in determining the parallel of longitude that intersects the center of the relief population. It was then a simple matter to translate the correction in miles to degrees, minutes and seconds.

The center of the relief population for October 1933 was found to be at 38°38′56″N, 86°4′2″W which is in south central Indiana, approximately 78 miles south of Indianapolis and about 94 miles northwest of Evansville (Figure I and Table II).

When the October 1933 data are grouped by states, it is possible to obtain an approximation to the relief center for any other month without much additional work. The sum of the products of the latitude distances and persons for each state, divided by the total number of persons on relief in the state gave the average latitude of persons on relief in that state. The same process was followed for the longitude. If the assumption is made that the center of the relief population of each state does not shift from month to month,[5] the center of the relief population of the United States can be readily obtained for any month by employing figures published monthly by the Federal Emergency Relief Administration. Proceeding as before, the sum of the products of the latitude of the center of each state multiplied by the number of persons on relief in that state, and divided by the total number of persons on relief in the country, yielded the latitude of the center for any month, and a similar procedure yielded the longitude of the center. For comparative purposes this procedure was followed for

[4] It should be noted that the value of a degree of latitude is always 69.0 miles, while the value of a degree of longitude varies with the latitude. Its value may be found from "Tables for a Polyconic Projection of Maps and lengths of Terrestrial Arcs of Meridians and Parallels," Fifth Edition, U. S. Dept. of Commerce, Coast and Geodetic Survey, Washington, 1930.

[5] The adequacy of this assumption is tested in the second part of this article.

May 1934, January 1935, May 1935 and October 1935.[6] Some important shifts have occurred during the period from October 1933 to October 1935. The center of the relief population moved westward and northward from October 1933 to January 1935 when it began to move eastward and in a northerly direction. In January 1935 the center of the relief population was west and north of the October 1933 center because of a smaller concentration of the relief population in the southeastern states. In October 1935 the center of the relief population was considerably north and slightly west of the October 1933 center. This was due to a large decrease in the relief load of both the southeastern and southwestern states and a somewhat smaller decrease in the New England states.

TABLE II

LOCATION OF CENTER OF RELIEF* POPULATION FOR CERTAIN SELECTED MONTHS; RELATIVE POSITION IN REGARD TO CENTER OF POPULATION, 1930; AND DISTANCE AND DIRECTION MOVED FROM PREVIOUS MONTH

Date	Location of Center		Distance in Miles from Center of Population, 1930			Distance Moved From Previous Month in Miles	
	Latitude	Longitude	Latitude	Longitude	Total Distance	Latitude	Longitude
Oct. 1933	38°38′56″	86° 4′ 2″	28.5 S	57.4 E	64.1	—	—
May 1934	39°10′31″	86°28′46″	7.8 N	35.3 E	36.2	36.3 N	22.1 W
Jan. 1935	39° 5′52″	87°33′51″	2.4 N	23.0 W	23.1	5.4 S	58.3 W
May 1935	39°12′41″	87°17′ 7″	10.3 N	8.0 W	13.0	7.9 N	15.0 E
Oct. 1935	39°28′20″	86°22′42″	28.3 N	40.8 E	49.7	18.0 N	48.8 E

* Includes general and special programs.

For only two of the five months considered were the special programs[7] of much importance. In Figure I and Table III the centers of the general and special relief program and for the general relief program are compared for January 1935 and May 1935. In both cases the center of the general relief population was northeast of the center of the general and special relief population. This was due to the fact that persons on special relief rolls were more heavily concentrated in the south and west than in other sections of the country.

Shifts in the center of the total population of the United States have been less extensive during the last 50 years than is true of the relief population during the last two years. During fifty years the center of the general population moved 133.1 miles west, nine miles north and nine miles south (Table I). In contrast, the relief population from October 1933 to October 1935 moved 80.4 miles west and then 63.8 miles east; and a total of 62.1 miles north and 5.3 miles south (Table II).

[6] Data for the first three months from Monthly Reports of the Federal Emergency Relief Administration. The October 1935 Data were taken from the monthly summary of Emergency Relief Statistics (mimeographed), released as preliminary figures by the Federal Emergency Relief Administration.

[7] Special programs included rural rehabilitation, emergency education, and college student aid. Of these rural rehabilitation was by far the most important in regard to number of persons benefited. This program began in April 1934 and in July 1935 its administration was transferred from the FERA to the Rural Resettlement Administration.

A STUDY OF ERRORS INVOLVED IN LOCATING THE CENTER OF THE RELIEF POPULATION

The obvious crudeness of the methods described above is regrettable but pains have been taken to study the main sources of error. In calculating the center of the relief population for October 1933 there were two main sources of error; first, the error in measuring the distance from the center of the county to the nearest latitude or longitude line, and second, the error in estimating the center of the county.

TABLE III

COMPARISON OF THE CENTER OF THE GENERAL AND SPECIAL RELIEF POPULATION WITH THAT OF THE GENERAL RELIEF POPULATION*

Date	Location of Center of General and Special Relief Population		Location of Center of General Relief Population		Distance in Miles from Center of General and Special Population to Center of General Population	
	Latitude	Longitude	Latitude	Longitude	Latitude	Longitude
Jan. 1935	39° 5′52″	87°33′51″	39°13′2″	87°30′43″	8.2 N	2.9 E
May 1935	39°12′41″	87°17′ 7″	39°25′8″	87° 2′34″	14.3 N	13.2 E

* Special programs were of appreciable size only from June 1934 to June 1935, so that the centers of the general and special relief population for October 1933, May 1934, and October 1935 were approximately the same as the centers of the general relief population.

The probable error involved in measuring from the center of the county to the nearest latitude or longitude line was found to be .0023 inches or .069 miles on the basis of the average map of 30 miles to an inch.[8] This was found by having a number of persons measure a fixed distance on a scale graduated in twenty-fifths of an inch, and then obtaining the probable error of the average measurement.

The amount of error in locating the center of the county was very small in counties which contained no disturbing factors, such as towns or cities, which prevented the geometric center from being the relief center, the probable error being only .012 inches or .36 miles. This was determined by separate measurements of ten irregular-shaped counties made by two persons, and the use of the customary probable error formula.

In order to determine the probable error of measurement of the center of a county whose center of population was not at the geometric center, measurements of the latitude and longitude of the centers of 57 counties in New York State were made by two persons. These counties being selected because their population and geometric centers do not coincide. The average distance between the centers located by the two persons was close to zero because the differences in direction tend to counterbalance each other. The probable error of the

[8] By use of the usual probable error formula. The average scale of maps was determined by weighting the scale of the map employed for each state by the relative influence of the state on the relief center.

distances between the centers of the 57 counties was .592 miles.[9] On the basis of the average state having a map of thirty miles to the inch, the probable error of the center of the average county of the country is .950 miles ($=.592\times30/18.7$ since New York has a scale of 18.7 miles to the inch). This is an extreme figure because there was a smaller error in locating the centers of large cities which were considered separately, and because about half the counties had their center at the geometric center, which is easier to locate accurately.

If it is assumed that the probable error of the center of each county is .950 miles, the probable error of the center of the relief population can be obtained by use of a simple formula.[10] The probable error proved to be .066 miles, or 350 feet, which is a maximum figure, because the assumptions mentioned were on the conservative side.

In order to test the validity of the assumption that the center of the relief population of a state remains unchanged from month to month, regardless of changes in the total relief loads of separate states, an analysis was made of the relief population of Alabama, Pennsylvania and Washington, in May 1935. The center of the relief population of each of these three states was calculated and compared to the center of October 1933 (Table IV). The center of the relief

[9] It is interesting to note, however, that the differences between the two sets of estimates of the location of the centers of counties produced a difference of only .07 miles in the location of the center of the state.

[10] Developed in the following way:

Let A_f denote the distance of the fth county from the center of population and N_f denote the number of persons in that county. Let a be the probable error for all counties of the distance to the center of the county from the center of population, and let n denote the total number of counties. In order to get the center of population we sum up the products of persons and distance from the center of population and divide by total persons. That is, the center of population is located by solving the formula

$$\frac{\sum_1^n N_f A_f}{\sum_1^n N_f}.$$

Knowing that A_f has a probable error of a for all values of f, we wish to know the probable error of the above expression. Since A_f has a probable error of a, then $N_f A_f$ has a probable error of $a \cdot N_f$. The sum of $N_f A_f$ for all values of f has a probable error of

$$\sqrt{\sum (aN_f)^2} = a\sqrt{\sum N_f^2}$$

since the probable error of the sum of any number of independent variables is the square root of the sum of the squares of their individual probable errors. Thus, the probable error of

$$\frac{\sum N_f A_f}{\sum N_f} \text{ is } \frac{1}{\sum N_f} \times a\sqrt{\sum N_f^2} = a\sqrt{\frac{\sum N_f^2}{(\sum N_f)^2}}$$

This reduces to $a\sqrt{\frac{1}{n}\left(1+\frac{\sigma^2}{\bar{N}^2}\right)}$ where $\bar{N}$ and σ are the arithmetic mean and standard deviation of the distribution of N's.

In the case of the relief population a was .950 miles; n, the number of counties, was 3097; $\bar{N}$, the average number of persons on relief in each county, was 4100; σ, the standard deviation of the distribution of counties by size of relief population, was 15,300. These give a probable error of .066 miles when substituted in the above formula.

TABLE IV

SHIFT IN CENTER OF RELIEF* POPULATION FROM OCTOBER 1933 TO MAY 1935 FOR THE U. S. AND THREE SELECTED STATES

Area	Relative Latitude**			Relative Longitude***			Total Change
	Oct. 1933	May 1935	Change	Oct. 1933	May 1935	Change	
U. S.	− 24.2	+ 28.9	+53.1	+ 3.6	+ 55.8	+52.2	74.5
Alabama	−421.4	−409.9	+11.5	+ 56.7	+ 50.0	− 6.7	13.3
Penna.	+119.9	+106.7	−13.2	− 478.2	− 447.2	+31.0	33.7
Wash.	+584.3	+579.0	− 5.3	+1669.6	+1671.4	+ 1.8	5.6

* Includes general relief program.
** Distance in miles from 39° N.
*** Distance in miles from 86° W.

population of the United States changed approximately 53 miles in latitude and 52 miles in longitude from October 1933 to May 1935, whereas the centers of the three states only changed +11.5, −13.2, and −5.3 miles, respectively, in latitude, and −6.7, +31.0, and +1.8 miles, respectively, in longitude. It thus appears that the shifts in the various state centers tended to have a compensating effect, with the result that the net effect of these shifts on the location of the relief center of the whole country would tend to be very small. In other words, the large shift in the relief center of the United States is due chiefly to external factors rather than the shifts occurring in the centers of the various states.

The total shifts for the centers of the three states were 13.3, 33.7, and 5.6 miles, respectively,[11] or an average of 17.5 miles. This average shift, if characteristic of the other states, would produce a maximum probable error of only 3.7 miles[12] in the relief center of the country for months other than October 1933. Since this probable error is much smaller than the month to month shifts shown in Tables II and III and on Figure I, it may be concluded that these shifts are significant rather than being due to accidental fluctuations in the individual states.

[11] Determined by extracting the square root of the sum of the squares of the latitude and longitude shifts.

[12] The formula was the same as that used for the center based on the county as the unit of estimate. Cf. footnote 10. To be conservative, it was assumed that the probable error of the relief center of each state was 17.5 miles (that is, the same as the average shift of the three states). The values for each of the symbols in the formula, with the state as the basis of estimate, were: $a = 17.5$ miles (the probable error of the center of the unit of estimate—here, the state); $n = 49$ (the number of states); $\bar{N} = 370{,}000$ (the average number of persons on relief in each state); $\sigma = 402{,}000$ (the standard deviation of the distribution of persons on relief in the various states).

THE SULFUR CONTAINING AMINO ACID METHIONINE

A Review of the Present State of Knowledge

By

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Among the sulfur compounds of living matter the structural unit of the amino acid cysteine has for some time received the lion's share of attention. Its ubiquitous presence in proteins, its oxidation to cystine *in vitro* and *in vivo* (2−SH→ −S−S−), its metabolic fate and presumed essential role, its tripeptide derivative glutathione, the specific biologic functions of its distinguishing −SH group, all have been the object of numerous studies, and as to the fundamental importance of these aspects for our growing understanding of the life process there remains no doubt. A compound which geneologically appears as a close relative of cysteine, while in its character and behavior it differs from it considerably, is the amino acid methionine which was discovered only in 1922. Recent developments seem to bear promise that as the study of this newcomer among the amino acids progresses, new and clarifying light will be thrown on some of the problems growing out of the cysteine-cystine studies, while some of our concepts on sulfur metabolism will need radical revision. The present review is the result of an attempt to coordinate and assess the available data relating to methionine in its various aspects.

CHEMICAL DATA

Discovery.—The discovery of methionine, by J. H. Mueller ('21-1, '21-2, '22-1, '22-2, '23-1) occurred more than a century after that of cystine (1810), and more than 40 years after the latter had been recognized as a protein constituent (1890). The presence in preparations of natural leucine of a sulfur containing impurity, which, in part at least, undoubtedly was methionine ('35-17, '36-3, '36-9), was early noted by Emil Fischer ('00-1) but apparently the observation was not followed up. Osborne ('02-1) found that the fraction of the total sulfur which is readily split off as inorganic sulfide by alkali varies greatly with different proteins. Table I shows that the percentage of "loosely bound sulfur" found by Osborne tends to run parallel with the percentage of cystine sulfur obtained by Baernstein's ('36-4) recent method, while Osborne's nonlabile sulfur is obviously related to the methionine sulfur. C. Th. Mörner ('14-1) by treating proteins with nitric acid obtained methylsulfonic acid and pointed out that the amount of the latter seemed to be inversely proportional to the cystine content. While thus evidence for the existence of protein sulfur other than that of cystine was not lacking, Mueller's discovery was not the result of research directed toward unknown forms of protein sulfur, but rather the product of studies on bacterial growth in meat infusions and the result of judicious follow-up of a casual observation on the loss of a nutrional factor by charcoal

treatment. Ironically the new substance was finally found not to be identical ('22-1) with the unknown growth factor which led to its discovery. The story of Mueller's discovery of methionine has been well summarized by Vickery and Schmidt ('31-5). The clarification of the structure of the compound we owe to Barger and Coyne ('28-1), who also proposed the present name.

Methods of Isolation.—The method of Mueller ('23-1) consists in neutralizing a sulfuric acid hydrolysate of casein with sodium hydroxide, decomposing a precipitate, formed by addition of ten per cent mercuric sulfate solution in five per cent sulfuric acid and succeeding neutralization by sodium hydroxide, with hot barium hydroxide solution, precipitating the filtrate from the barium sulfate and mercuric oxide, after removal of barium and sulfate ions, with a hot saturated mercuric chloride solution, again decomposing the resulting precipitate by hot barium hydroxide, evaporating the filtrate, and, after removal of chloride ion by silver oxide treatment, allowing the concentrated hot aque-

TABLE I. COMPARISON OF THE DATA OF OSBORNE ('02-1) AND BAERNSTEIN ('36-4) ON SULFUR IN PROTEINS

Protein	"loosely bound sulfur" (Osborne)	Cystine Sulfur (Baernstein)	Methionine Sulfur (Baernstein)	H_2S-Sulfur (Baernstein)	Unaccounted Sulfur (Baernstein)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Edestin (hemp)	40	38	48	15	0
Vitellin	34	31	49	12	8
Egg Albumin	30	34	60	5	2
Casein	13	10	83	2	4

ous solution of the evaporation residue to crystallize after addition of 3 to 4 volumes of alcohol. By again precipitating the solution of the resulting crystals with mercuric chloride and working up as before, crystals corresponding to the composition $C_5H_{11}SNO_2$ were obtained in 0.2 to 0.4 per cent yield. Odake ('25-1), in isolating a very small amount of methionine from yeast used hydrogen sulfide for the decomposition of the mercury precipitates, but otherwise followed the principles of isolation established by Mueller. By two mercuric chloride precipitations and two recrystallizations from 60 to 80 per cent alcohol he could isolate, from an amino acid mixture which chiefly consisted of leucine and which, according to its sulfur content, contained a maximum of 11 per cent methionine, 46 per cent of this amount in analytically pure form. Barger and Coyne's ('28-1) experiments indicate that the precipitate formed by mercuric sulfate in the presence of free sulfuric acid contains other amino acids, while the mercury compound of methionine precipitates only on neutralization. They obtain from casein a yield of 0.1 to 0.3 per cent of 85 to 95 per cent pure methionine after only one mercuric chloride precipitation, omitting the second one, since it "is very wasteful." Du Vigneaud and Meyer ('32-13) isolated 0.07 per cent of pure methionine from a pancreatic hydrolysate of casein, by allowing tyrosine to crystallize spontaneously from the neutral solution, precipitating a tryptophane-histidine fraction by mercuric sulfate at acid reaction, and finally the methionine by mercuric sulfate in a neutral range. This mercury

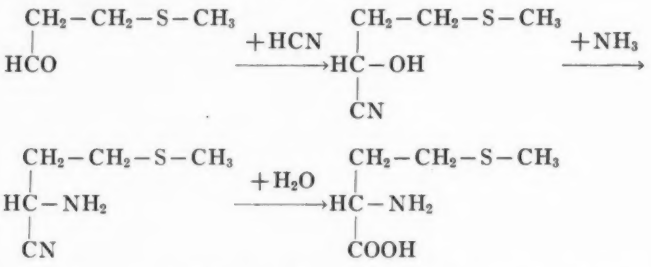
precipitate, after removal of barium and sulfate ions, was further purified by precipitation with mercuric chloride from an acidified solution. The mercuric chloride precipitate was washed with ice water, decomposed with hydrogen sulfide, the filtrate from mercuric sulfide was evaporated in vacuo, the residue which contains the amino acid in the hydrochloride form was taken up in alcohol, decolorized by charcoal and the free amino acid was caused to precipitate by the addition of aniline. Final purification was effected by precipitating with alcohol from a concentrated aqueous solution.

The yield from casein was raised to 1.0–1.4 per cent of 95 per cent pure methionine by a method published by Pirie ('32-9). After distilling off excess HCl from a hydrochloric acid hydrolysate and precipitation of the solution with lead acetate—to remove some of the impurities—the acid filtrate was extracted with butyl alcohol. The extracted monoamino acids were removed from the butyl alcohol by extraction with aqueous alkali. The aqueous solution is concentrated, filtered, neutralized with hydrochloric acid and precipitated by mercuric acetate in 3 per cent acetic acid solution. The resulting precipitate is decomposed by barium hydroxide and the filtrate, after evaporating to a small volume, in order to remove hydrochloric and acetic acid, is poured into a hot mercuric chloride solution. The precipitate filtered after cooling to freezing temperature, is decomposed by hydrogen sulfide, the evaporated filtrate is taken up in alcohol and the free amino acid precipitated by addition of pyridine. To insure a good yield excess hydrochloric acid was removed as completely as possible in the preceding evaporation as the solubility of methionine in the alcoholic medium was found to be considerably increased by the presence of pyridine hydrochloride. By the same method Pirie isolated 2.5 per cent of methionine from egg albumin ('32-10). Subsequently ('33-8) Pirie proposed a different approach, in which he used an enzymatic casein hydrolysate, chiefly in order to eliminate chloride or sulfate ions, since he found that both have an "inhibiting effect" in the mercury precipitation. The neutralized hydrolysate is directly precipitated by a combination of mercuric acetate and phosphotungstic acid, since the latter, even though not a precipitant for methionine by itself, was found to increase the efficiency of the mercuric acetate precipitation. The resulting precipitate is decomposed by barium hydroxide, and from the filtrate, after evaporating to a small volume, an amino acid mixture containing about 28 per cent of methionine is obtained by addition of alcohol. From this the pure compound is isolated by mercuric chloride precipitation as in the preceding method. No improvement in yield, but technical advantages are claimed for the new method. It is not recommended for use with egg albumin as the starting material on account of difficulties in its enzymatic digestion. A new departure was presented by Hill and Robson ('34-8) who discovered that the isoelectric solubility of methionine is considerably lowered in a saturated sodium chloride solution. From a hydrochloric acid hydrolysate of casein or egg albumin excess acid is removed by distillation, the residue taken up in water is decolorized by charcoal at a pH of about 4, and tyrosine is allowed to crystallize. The filtrate is concentrated to incipient crystallization of sodium

chloride and the methionine is obtained from an amino acid fraction which separates out at this stage. The chief admixture is leucine. Purification is effected by a first precipitation with mercuric acetate followed by sodium chloride which, if added in optimal amount, causes an increase in the mercuric acetate precipitate. However, care is necessary, as an excess of either mercuric acetate or sodium chloride will redissolve the precipitate. The latter is decomposed by hydrogen sulfide and the methionine is obtained pure by further precipitation with mercuric chloride, and final precipitation from alcohol by pyridine as recommended by Pirie. The reported yield is 1.5 per cent from casein and 2.5 per cent from egg albumin.

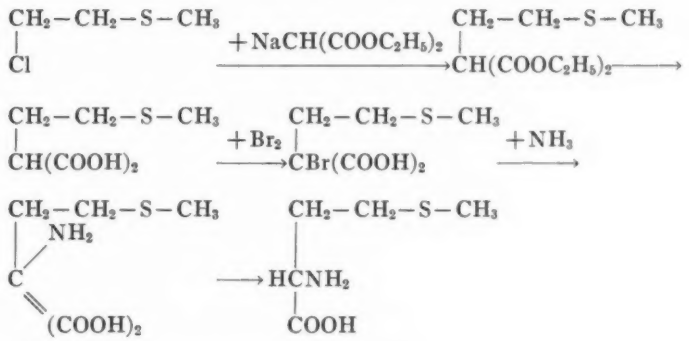
The purity, analytical or stereochemical, of the resulting product is not stated in most of the publications dealing with the isolation of natural methionine. The chief contaminants, whose complete removal offers difficulties, appear to be phenylalanine, glutamic acid ('23-1, '32-1) and leucine[1] ('25-1, '34-8). Ester distillation ('23-1, '28-1), copper salt precipitation ('25-1) and resolution of the synthetic dl-compound ('34-8) have been considered and rejected in connection with the preparation of the natural compound. Among the ill-understood features of the various isolation methods are the chemical nature of the precipitates formed with different mercury salts, the mechanisms responsible for the reported increase of the mercuric acetate precipitation by sodium chloride ('34-8) or by phosphotungstic acid ('33-8) and the redissolving effect of chloride as well as of sulfate ion ('33-8). Apparently it is the mercuric chloride molecule which is the actual precipitant ('25-1, '28-1, '34-8, '35-17). Therefore, the formation of the latter (which is characterized by its very low degree of dissociation) when mercuric ion in the form of mercuric acetate is combined with a hydrolysate containing chloride ion appears as an essential step, and the disappearance of this ($HgCl_2$) molecular species by the reaction $HgCl_2+2Cl^-\rightarrow[HgCl_4]^=$ also requires consideration. Some of the phenomena noted can be explained on the basis of these reactions.

Synthesis.—The first synthesis of methionine, by which its structure was proved, is that of Barger and Coyne ('28-1)



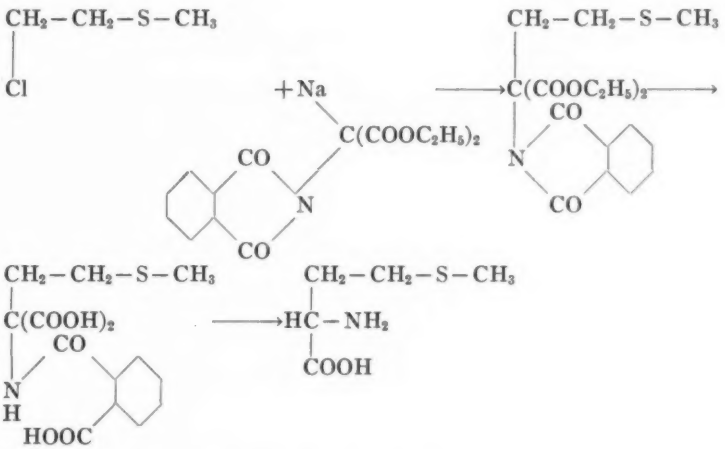
The yield in terms of the aldehyde was about 6 per cent. An alternate, but hardly superior method was proposed by Windus and Marvel ('30-4).

[1] Conversely the difficulty of obtaining leucine preparations free of methionine has been noted ('35-17, '36-3, '36-9, '37-9, '37-25).

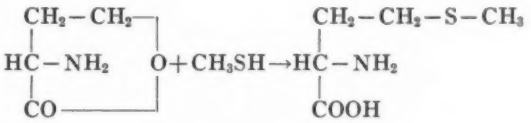


The yield was 8%. Some improvements were contributed by Emerson, Kirk and Schmidt ('31-3).

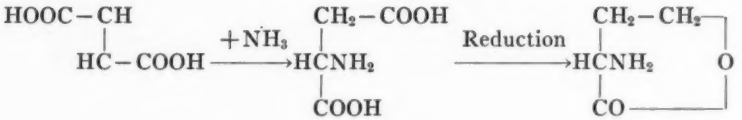
A more efficient synthetic path is the one followed by Barger and Weichselbaum ('31-1, '34-2).



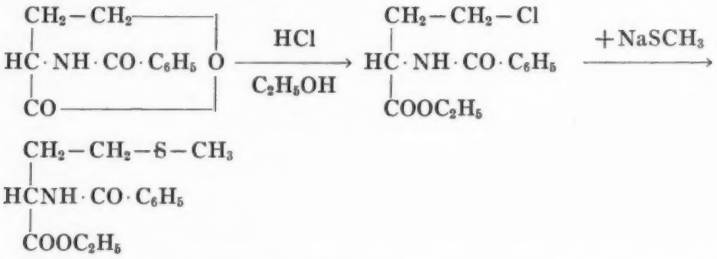
where the comparable yield is about 58%. Still another approach was shown by Hill and Robson ('36-11) who considered the possibilities of a biological synthesis according to



in which the α-amino γ-butyrolactone might be related to fumaric and aspartic acid as follows:

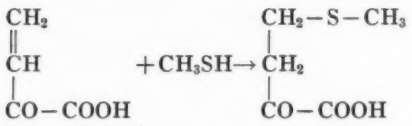


As the nearest comparable process *in vitro* the authors achieved the synthesis by the following intermediary steps:



with a final yield of methionine of 31% in terms of the benzamido-butyrolactone.

The possibility of a biological synthesis of methionine by a different kind of mercaptan addition has been discussed by Nicolet ('35-18): addition of methylmercaptan to the hypothetical methylene pyruvic acid would yield a keto derivative of methionine which might be an intermediary in its formation or metabolism:



Properties and Constants.—Methionine like other amino acids has no well defined reversible melting point. The point of decomposition and liquefaction as reported by different authors, is 267–270° ('34-12), 272–3° ('25-1), 275–7° ('32-1) and 283° (uncorr.) ('23-1) for the natural (l-) form, and 272° (uncorr.) ('30-4), 275–6° (corr.) ('33-12), 279–80° (corr.) ('31-1, '34-2) and 281° ('28-1) for the synthetic (dl-) compound.

Microphotographs of the crystals of the natural compound, described as "shiny monoclinic plates," have been published ('25-1). Mueller ('23-1) speaks of "massed hexagonal plates," while the synthetic compound has been described as "clusters of narrow platelets" ('28-1).

An ebullioscopic molecular weight determination, in a 1.5 per cent aqueous solution gave a value within 6 per cent of the one calculated ('23-1). Curves relating solubility and pH in water and saturated sodium chloride solution at room temperature have been shown for l-methionine (the purity of which is not stated) by Hill and Robson ('34-8); the isoelectric solubility of about 5 grams per 100 cc. is depressed to less than one-half of this value by saturation

with sodium chloride. Dalton and Schmidt ('35-8) made exact measurements of the solubility in water from 0° to 100° of the dl-compound: the values range (grams per 100 grams of water) from 1.82 at 0° to 17.6 at 100°, while the 25° value is about 3.4. A determination of the dissociation constant of the dl-compound is also on record ('31-3): pK′a = 9.21 and pK′b = 11.72, so that 5.74 is the pH of the isoelectric point.

No systematic study of the optical rotation has as yet been reported. The available scattered data have been assembled in Table II.

The substances referred to in this table were obtained from acid hydrolysates of casein (data no. 1, 6, 7), of horn (no. 8), of yeast (no. 9), from an enzymatic digest of casein (no. 4), by resolution of the synthetic compound (no. 2, 3, 10, 11, 13, 15) and synthetically from resolved synthetic homocysteine (no. 5, 12, 14). The precision of most of these data is hardly better than ±5 to 10 per cent. The most obviously conflicting value in the series is the one of Odake (no. 9) although the careful data of his paper suggest a product of high purity. However, the large pH effect which is evident will require consideration.

TABLE II. THE OPTICAL ROTATION OF METHIONINE

No.	Compound	Medium	Concentration	Estimated* pH	Temperature	$[\alpha]_D$	Reference
1	l-methionine	H_2O	0.8%	5.8	25°	−6.9°	'31-7
2	l-methionine	H_2O	0.8%	5.8	25°	−7.5°	'31-7
3	l-methionine	H_2O	0.8%	5.8	25°	−8.1°	'31-7
4	l-methionine	H_2O	0.8%	5.8	25°	−7.3°	'32-13
5	l-methionine	H_2O	1.0%	5.8	25°	−7.7°	'35-22
6	l-methionine	H_2O	3.0%	5.8	21°	−7.9°	'32-9
7	l-methionine	H_2O	2.8%	5.8	20°	−7.2°	'23-1
8	l-methionine	H_2O	5.0%	5.8	20°	−7.3°	'32-1
9	l-methionine	H_2O	2.0%	5.8	16°	−11.8°	'25-1
10	d-methionine	H_2O	0.8%	5.8	25°	+8.8°	'31-7
11	d-methionine	H_2O	0.8%	5.8	25°	+8.1°	'31-7
12	d-methionine	H_2O	1.0%	5.8	25°	+8.0°	'35-22
13	d-methionine	0.2 M HCl	0.8%	0.9	25°	−21.2°	'31-7
14	d-methionine	0.2 M HCl	1.0%	0.9	25°	−22.0°	'35-22
15	d-methionine	0.6 M $NaHCO_3$	0.8%	8.4	25°	−7.5°	'31-7

* By the reviewer.

Reactions and Reactivity.—Methionine behaves like a genuine amino-carboxylic acid in displaying the characteristic ninhydrin color reaction ('25-1). As an amino acid it is susceptible to racemization of its optically active modifications. However, in acid solutions this may practically not be a serious factor, inasmuch as methionine isolated from an enzymatic hydrolysate ('32-13) did not show a higher specific rotation than that resulting from hydrolysis by extended boiling with hydrochloric acid. By comparison racemization proceeds easily under alkaline conditions, since methionine obtained by alkaline protein hydrolysis was optically inactive ('23-1).

On account of its metabolic implications the stability of the –C–S– linkages and the formation of sulfate ion from the compound has been studied in a number of ways. The surprising stability on alkaline boiling of the sulfur linkages of

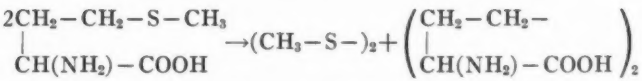
methionine compared with those of the isomeric ethyl−S−cysteine, which was noted by the early investigators ('23-1, '25-1), will be discussed in the section on derivatives. Even under the influence of violent oxidizing agents the methionine-sulfur appears strongly protected against complete conversion to sulfate.[1] Blumenthal and Clarke ('35-4) showed that methionine, in common with cystine, forms no sulfate under the influence of bromine water, while even on 36 hours' boiling with concentrated nitric acid—conditions under which the total sulfur of cystine is easily converted—only minor amounts of sulfate are formed. The same difference is noted in the action of nitrous acid which under the conditions of the Van Slyke amino nitrogen determination may form considerable amounts of sulfate from cystine ('34-9, '37-11), while none is produced from methionine ('33-3, '34-9). That , in contrast with cystine, methionine gives the correct nitrogen value in the Van Slyke method was already noted by Mueller ('23-1). Even under the conditions of the Benedict-Denis sulfur determination method where oxidation is effected by boiling and fusing with cupric nitrate, less than 40 per cent of the total sulfur has been obtained as sulfate ('36-16). Low values have also been reported for the homologous methyl−S−cysteine ('37-7), and for the total sulfur content of proteins ('37-1), where it probably is due to their relatively high methionine content (cf. Table III). However, in the case of methionine, the conversion could be increased to 95 per cent ('37-18) by bringing the moderately acid (pH 3, cf. '32-14) Benedict-Denis reagent to a neutral reaction but also by using the strongly acid oxidizing reagent of Pirie ('32-10) which consists of copper nitrate, nitric acid and perchloric acid. The formation of methylmercaptan ('23-1; "boiled cabbage" odor, as well as evolution of SO_2 was noted on dry heating; '36-11) and its oxidation products is undoubtedly a factor in these incomplete oxidations: dimethyldisulfide ('32-5) or dimethyldisulfoxide (formed from the disulfide by dilute nitric acid, volatile with water vapors, according to Beilstein's Handbook) which may be lost by volatilization, or methylsulfonic acid which is extremely resistant to hydrolytic separation of the methyl group ('14-1, '31-6).

The reaction potentialities are further illuminated by investigations dealing with the action of strong sulfuric acid and hydriodic acid. By heating a solution of dl-methionine in 60 per cent sulfuric acid for 8 hours at about 130° in a nitrogen atmosphere Butz and du Vigneaud ('32-5) obtained, in 43 per cent yield, a homolog of cystine which they named homocystine. The simplest picture for the reaction involved would be

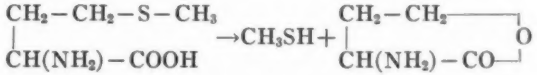
$$\begin{matrix} 2CH_2-CH_2-S-CH_3 \\ | \\ CH(NH_2)-COOH \end{matrix} \rightarrow \left(\begin{matrix} CH_2-CH_2-S- \\ | \\ CH(NH_2)-COOH \end{matrix} \right)_2 + C_2H_6$$

However, identification of ethane was not attempted. On the other hand the yield and the presence of the odor of dimethyldisulfide indicate that in this acid decomposition various reactions run parallel, one of which may be

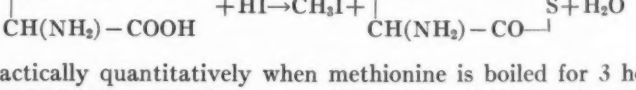
[1] The contrasting ease of sulfate formation *in vivo* will be dealt with in the biological part.



involving formation of a hypothetical new diaminodicarboxylic acid. Another possibility was suggested by Hill and Robson ('36-11). Noting an odor which they attributed to methylmercaptan, when methionine was treated with strong sulfuric acid in the Kjeldahl method, they considered the possibility of the formation of aminohydroxybutyric acid which, as a γ-hydroxyacid, should appear as a lactone:



Direct evidence for this reaction as well as for its conceivable reverse is lacking. However, an alternative decomposition involving demethylation ('28-1) has been studied and analytically utilized by Baernstein ('32-2, '34-1). He showed that the reaction



occurs practically quantitatively when methionine is boiled for 3 hours with 57 per cent hydriodic acid. Hydrogen sulfide and methylmercaptan have been identified as by-products in this reaction ('37-10).

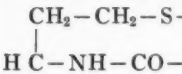
Salts and other Derivatives.—The only well defined compound with inorganic cations that has been described is the cupric salt which crystallizes in light blue minute hexagonal plates, is almost insoluble in cold and moderately soluble in hot water ('23-1), while its color is also described as "light blue-violet," its solubility in hot water is reported as 1:6000, and its composition as that of the normal salt $(C_5H_{10}SNO_2)_2Cu$ ('25-1). A mercury-mercuric chloride compound of apparently complex nature has been utilized, since Mueller first described it ('23-1) in the isolation of methionine. In order to obtain it in granular, rather than oily, form it should be prepared by allowing a dilute and hot solution to cool (the compound tends to dissolve in the heat). Its special virtue in the separation of methionine from leucine has been variously recommended ('25-1, '28-1, '34-8, '35-17). The only analysis of this complex, by Mueller ('23-1), may indicate a structure $[CH_3-S-CH_2-CH_2(NH_2)-COO-]_2Hg+4HgCl_2$, i.e. a mercuric salt of methionine in which each methionine unit is combined with two mercuric chloride units, possibly by coordinate linkage at the sulfur atoms.[2] Among the salts with acids the hydrochloride, which crystallizes from strong hydrochloric acid, has been described ('32-9), while the absence of precipitate formation by phosphotungstic acid ('25-1, '33-8) and by picric acid ('25-1) has been recorded. The picrolonate (soluble in water and

[2] For mercuric chloride compounds of the type $R_1-S-R_2+2HgCl_2$, derived from thiophen and other sulfides, cf. ('14-2, '29-1, '34-4).

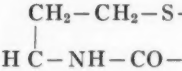
alcohol, but crystallizing from acetone) has been utilized for identification purposes ('28-1, '30-4).

Among esters of methionine only the methyl-ester hydrochloride of the racemic compound has been described as a well defined product ('31-1) while passing references to the ethylester (soluble in ether, little soluble in water; distilling together with that of phenylalanine; unstable on distillation even in high vacuum) are found in connection with isolation experiments ('23-1, '28-1).

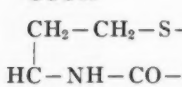
A number of compounds representing substitutions in the amino group have been prepared. N−methyl−methionine was obtained by a synthetic method ('36-17), N−formyl−methionine and N−benzoyl−methionine were obtained by formylation and benzoylation of racemic methionine, the brucine salts of the d- and l−N−formyl compound have been used for the resolution of synthetic methionine ('31-7) and N−formyl−l−methionine was also prepared ('31-7). In addition, the following amino derivatives have been described:



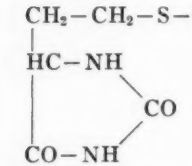
(dl-)carbamido-methionine ('36-11)



α-naphthylcarbamido-methionine, derived from the natural compound ('23-1, '25-1, '28-1, '31-7, '34-12) and from the dl-form ('28-1).



(1-)p-tolylcarbamido-methionine ('31-7)



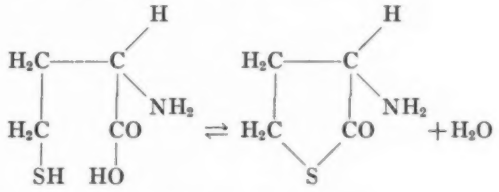
(dl-)methionine hydantoin ('28-1, '36-11)

Preparation of the zinc salts of d- and l-α-hydroxy-γ-methiobutyric acid, resulting by replacing the amino group of methionine with hydroxyl has been described by Akobe ('36-1). The action of B. oidium lactis on l-methionine furnished the d-hydroxy derivative, while another organism, B. subtilis, produced the l-hydroxy-compound. The latter was also obtained by deamination of l-methionine with nitrous acid.

The most direct functional interest attaches of course to those derivatives of methionine that are the result of variations in the sulfur grouping.

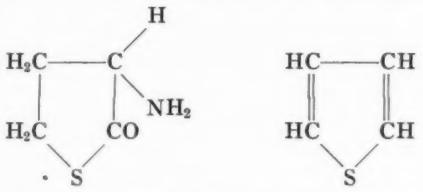
A sulfoxide would result from addition of one oxygen atom, while a second one would form a sulfone. These compounds have been discussed ('28-1, '32-10)

but no preparation has been reported. Furthermore, methionine may be considered as the S-methyl ether of a thiolcompound which is the next higher homolog of cysteine. This latter compound has been prepared, and named homocysteine, by Riegel and du Vigneaud ('35-19). It is of great interest chemically and biologically because it is distinct in its chemical properties from cysteine even though in its composition it differs by only one $-CH_2-$ group. The available data indicate that a reversible equilibrium exists between homocysteine and its thiolactone, or inner anhydride:



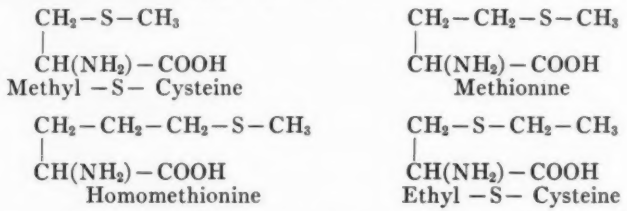
This thiolactone was discovered by Baernstein ('34-1) who obtained it in the form of its hydriodide in the demethylation of methionine by concentrated hydriodic acid. The thiolactone ring is opened by the action of alkali; however, the alkalinity of the Folin-Marenzi sulfhydryl test ('29-2) is not sufficient so that the compound escapes determination in this method, while slow color evolution under the action of ammonia and nitroprusside shows that the $-SH$ group is being gradually liberated ('34-1). On the other hand increasing acidity favors closure of the ring ('35-19) so that the sulfhydryl group in the thiolactone form is protected against oxidation to the disulfide by iodine in acid solution ('36-3). The oxidation is made possible by the opening of the ring in alkaline medium. This fact has been utilized by Baernstein in his analytical method for distinguishing cysteine and the thiolactone ('36-3). The former is oxidized to cystine by iodine in acid medium and subsequently the latter is oxidized to homocystine by tetrathionate ion in alkaline solution. That the velocity of the reversible reaction is relatively slow outside of the ranges of extreme acidity and alkalinity is indicated by the observations of Baernstein ('34-1, '36-3) and of Riegel and du Vigneaud ('35-19), who showed that even in 0.1 N hydrochloric acid at boiling temperature the ring closure reaction had a half-time of about three hours, and also by work of Brand and associates ('35-5) who studied the reduction of homocystine and found that by catalytic reduction with hydrogen in normal hydrochloric acid no free $-SH$ was obtained even though the calculated amount of hydrogen was consumed. Reduction with zinc in 1.4 N HCl furnished approximately the calculated amount of sulfhydryl. It may be surmised, in connection with the data of Riegel and du Vigneaud ('35-19) that in the slower catalytic method reduction was accompanied by ring closure, while in the other method the greater rapidity and immediate neutralization prevented the cyclization. A closer study of the equilibrium between homocysteine and its thiolactone seems indicated on account of its probable biological significance. The thiolactone structure may be looked upon as a protected latent

form of the biologically important and at the same time oxidation-sensitive sulfhydryl group. The buffering action involved in the conversion may also be of interest: because of the disappearance of the carboxyl group the amphoteric thiolcompound turns into the basic thiolactone by the loss of water which apparently is favored by increase in acidity. At the same time the structural relation between the thiolactone and the coal tar constituent thiophen



may be significant with regard to the biogenetic origin of the latter. Physiological occurrence of the thiolactone has not been demonstrated but it seems probable, and an indication if its presence in urine after methionine feeding may be seen in an observation of Chase and Lewis ('33-3) who found a positive test with nitroprusside and ammonia that reached its maximum after seven minutes. Free $-SH$ produces its maximum color immediately under these conditions while the behavior of the thiolactone as described by Baernstein ('34-1) is similar to the observation of Chase and Lewis.

Among other close structural relatives of methionine methyl$-$S$-$cysteine, homomethionine and ethyl$-$S$-$cysteine are of interest. The first compound differs from methionine by the absence of one CH_2 group, the second by the presence of an additional CH_2 group, while the third differs only in the relative position of the two adjoining groups CH_2 and S:



l-Methyl$-$S$-$cysteine was prepared by two different methods (reduction of cystine by sodium in liquid ammonia and reaction with methyl iodide; methylation of cysteine with dimethylsulfate in alkaline solution) by du Vigneaud, Loring and Craft ('34-15). They report a decomposition point of 248° and a specific rotation of $[\alpha]_D^{27} = -31°$ in a 1% solution in water. Brand, Block and Cahill ('37-7) made the same compound from cystine by reduction with tin and hydrochloric acid followed by reaction with dimethylsulfate in alkaline solution (method of Clarke and Inouye ('31-2)) and found the decomposition point at 248° and $[\alpha]_D^{29} = -23°$.

Homomethionine has been prepared in the racemic form by du Vigneaud,

Dyer, Jones and Patterson ('34-14) by a synthetic method. It showed solubility relations in water and alcohol similar to those of methionine and a decomposition point of 272–274° (corr.).

l-Ethyl−S−cysteine has been described by Brenzinger ('92-1), by Neuberg and Mayer ('05-1), and by Clarke and Inouye ('31-2). It was prepared by reaction of a mercury mercaptide of cysteine with ethyliodide ('92-1, '05-1) and by reduction of cystine with tin in hydrochloric acid followed by reaction with ethylsulfate in alkaline solution ('31-2). The decomposition points as reported are contradictory: 226–228° ('92-1), 164–166° ('05-1) and 260° ('31-2). The following specific rotations have been given:

$[\alpha]_D = -28.3°$, 5% in H_2O ('92-1)
$[\alpha]_D^{25} = -23.7°$, 3.2% in H_2O ('31-2)
$[\alpha]_{Hg}^{23} = -27.4°$, 3.2% in H_2O ('31-2)
$[\alpha]_D = -11.8°$, 8% in 0.54 N HCl ('92-1)
$[\alpha]_{Hg}^{24} = -3.8°$, 2.8% in N HCl ('31-2)
$[\alpha]_{Hg}^{24} = +5.2°$, 3.3% in N NaOH ('31-2)

These constants are of some interest for comparison with those of the isomer methionine. In chemical behavior the two compounds show very definite differences. Brenzinger ('92-1) showed that ethyl−S−cysteine is decomposed, with formation of ethylmercaptan and ammonia, by sodium hydroxide under comparatively mild conditions. This decomposition has been investigated quantitatively by Clarke and Inouye ('31-2) who found that the evolution of ammonia in boiling normal sodium hydroxide has a reaction half-time of about 20 hours, indicating a rather higher stability than that suggested by the report of Brenzinger ('92-1). However, the formation of mercaptan was not measured. Mueller ('23-1) upon comparing methionine with ethyl−S−cysteine prepared according to Brenzinger ('92-1) concluded that methionine is "apparently quite stable" when treated with alkali under conditions which lead to evolution of mercaptan and ammonia in the case of ethyl−S−cysteine. This was confirmed by Odake ('25-1) who obtained no reaction for −SH when methionine was strongly heated with concentrated sodium hydroxide. A quantitative comparison of the stability of the two isomers, which would be of interest in view of the metabolic behavior of the two compounds, is lacking.

BIOLOGICAL DATA[3]

The literature dealing with the biological aspects of methionine will be considered under the following sub-groups: (a) occurrence in the biological world, (b) rôle in normal adult metabolism, (c) rôle in growth, (d) pathological and other special metabolic aspects, and (e) biological difference between d- and l-methionine and position in comparison with derivatives.

Occurrence.—The available quantitative data on the methionine content of proteins of various kind and origin have been obtained by the two methods of

[3] The stereochemical prefixes dl-, l- or d- have been used in connection with methionine whenever it has been evident from the original text which form has been used.

TABLE III. THE METHIONINE CONTENT OF PROTEINS

	Total Sulfur	Methionine	Fraction of Total Sulfur Present as Methionine	Reference
	per cent	*per cent*	*per cent*	
Plant Proteins				
adsuki-bean, α-globulin (Jones)	0.88	2.37	58	'32-2, 3[a]
adsuki-bean, α-globulin (Jones)	0.88	2.09	51	'36-3[a]
adsuki-bean, α-globulin (Jones)	0.88	2.02	49	'36-3[a]
adsuki-bean, β-globulin (Jones)	0.40	1.22	66	'32-2, 3[a]
arachin (globulin from peanut, Jones)	0.42	0.54	28	'32-2, 3[a]
cocksfoot (grass)	1.01	1.7	35	'37-1[b]
coconut globulin (Jones)	1.04	2.05	42	'32-2, 3[a]
conavalin (jack bean, Jones)	0.51	1.81	76	'32-2, 3[a]
dicoccumin (Gortner)	1.20	2.78	50	'32-2, 3[a]
edestin (hemp)	0.99	2.07	45	'32-2, 3[a]
edestin (hemp)	0.99	2.39	52	'34-1[b]
edestin (hemp)	0.99	2.20	48	'36-3, 4[b]
edestin (hemp)	0.93	2.3	53	'37-1[b]
edestin (Pearson)	0.62	1.35	47	'36-3[a]
edestin (Pearson)	0.62	1.44	50	'36-3, 4[b]
gliadin (Gortner)	0.99	2.03	44	'32-2, 3[a]
gliadin	1.19	1.6	29	'37-1[b]
glycinin (soy bean, Jones)	0.84	1.84	47	'32-2, 3[a]
hordein (Gortner)	0.90	2.24	54	'32-2, 3[a]
kafirin (Gortner)	0.59	1.61	59	'32-2, 3[a]
lentils albumin	0.82			'35 20[d]
lima-bean, α-globulin (Jones)	1.15	2.31	43	'32-2, 3[a]
lima-bean, β-globulin (Jones)	0.35	0.73	45	'32-2, 3[a]
monococcumin (Gortner)	1.14	3.04	57	'32-2, 3[a]
sativin (Gortner)	1.63	3.93	52	'32-2, 3[a]
secalin (Gortner)	1.26	1.51	26	'32-2, 3[a]
sorghumin (Gortner)	0.56	1.76	68	'32-2, 3[a]
speltin (Gortner)	1.20	2.61	47	'32-2, 3[a]
stizolobin (Jones)		1.94		'32-2[a]
teozein (Gortner)		3.25		'32-2[a]
tomato seed, α-globulin (Jones)	0.97	3.14	70	'32-2, 3[a]
tomato seed, β-globulin (Jones)	0.79	1.72	47	'32-2, 3[a]
yeast		present (isolated)		'25-1[c]
zein (Harris)	1.08	2.58	51	'36-3[a]
zein (Harris)	1.08	2.46	49	'36-3, 4[b]
zein (Gortner)		2.21		'32-2[a]
zein (Jones)	0.93	2.35	54	'32-2, 3[a]
zein (Jones)	0.93	2.32	54	'36-3[a]
zein (Jones)	0.93	2.25	52	'36-3[b]
Animal Proteins				
Egg Proteins				
ovalbumin	1.61	4.57	61	'32-2, 3[a]
ovalbumin	1.61	5.07	68	'36-3[a]
ovalbumin	1.61	5.24	70	'34-1[b]
ovalbumin	1.61	4.49	60	'36-3, 4[b]
ovalbumin (Merck)	1.64	3.84	50	'36-3[a]
ovalbumin (Merck)	1.64	3.55	47	'36-3, 4[b]
ovalbumin (Merck)	1.83	5.29	62	'32-2, 3[a]
ovalbumin (Merck)	1.83	5.10	60	'34-1[b]
ovomucoid (Jukes)	2.26	1.43	14	'36-3[a]
ovomucoid (Jukes)	2.26	1.38	13	'36-3, 4[b]
livetin (Jukes)	1.80	2.33	28	'36-3[a]
livetin (Jukes)	1.80	2.35	28	'36-3, 4[b]
vitellin (Jukes)	1.14	2.70	51	'36-3[a]
vitellin (Jukes)	1.14	2.60	49	'36-3, 4[b]
yolk albumin		1.94		'35-20[d]

TABLE III. THE METHIONINE CONTENT OF PROTEINS—*Continued*

	Total Sulfur	Methionine	Fraction of Total Sulfur Present as Methionine	Reference
	per cent	*per cent*	*per cent*	
fibrin	0.97	2.40	53	'32-2, 3[a]
fibrin	0.97	2.59	57	'34-1[b]
fibrin (Gortner)	0.97	2.37	53	'32-2, 3[a]
gelatin (Swift, Jones)	0.47	0.97	44	'32-2, 3[a]
insulin[e]	3.13	0.7	5	'36-13[b]
Keratins				
horn		present (isolated)		'32-1[c]
wool	3.6	0.5	3	'33-1[a]
wool	3.0–4.0	0.44–0.67	2.4–2.8	'34-3[a]
wool	3.3–3.7	0.6–0.8	3.5–5.1	'37-1[b]
Milk Proteins				
casein	0.90	3.53	84	'32-2, 3[a]
casein	0.90	3.50	84	'34-1[b]
casein (Harris)	0.80	3.31	89	'36-3[a]
casein (Harris)	0.80	3.10	83	'36-3, 4[b]
casein (Gortner)	0.80	3.36	90	'32-2, 3[a]
casein (Jones)	0.83	3.25	84	'32-2, 3[a]
casein cow's milk	0.65	2.89	87	'37-16[b]
casein human milk	0.78	2.70	74	'37-16[b]
lactalbumin (Harris)	1.45	2.45	36	'36-3[a]
lactalbumin (Harris)	1.45	2.32	34	'36-3, 4[b]
lactalbumin (Jones)	1.56	2.63	36	'32-2, 3[a]
lactalbumin (Jones)	1.56	2.62	36	'34-1[b]
lactalbumin cow's milk	1.52	2.29	32	'37-16[b]
lactalbumin human milk	1.65	1.40	18	'37-16[b]
beef muscle protein (Jones)		3.66		'32-2[a]
halibut muscle protein (Jones)	1.16	3.98	74	'32-2, 3[a]
shrimp muscle protein (Jones)	1.25	3.41	59	'32-2, 3[a]
myogen (albumin), rabbit muscle	1.29	2.8	47	'37-2[b]
myosins (globulin)				
chicken	1.12	3.43	66	'37-2[b]
dog	1.06	3.35	68	'37-2[b]
fish	1.21	3.65	65	'37-2[b]
lobster	1.21	3.45	61	'37-2[b]
rabbit	1.10	3.4	57	'37-2[b]
peptone (Witte's)	0.89	2.19	53	'36-3[a]
peptone (Witte's)	0.89	2.20	53	'36-3, 4[b]
beef protein		0.21		'35-20[d]
beef albumin		1.06		'35-20[d]
fish albumin		3.25		'35-20[d]
sardine muscle protein		0.52		'34-12[c]
whale muscle protein		0.37		'34-12[c]
silk worm pupa protein		0.43		'34-12[c]

[a] Baernstein's first method ('32-3).

[b] Baernstein's second method ('34-1, '36-3).

[c] Stated amount has been isolated.

[d] Method unknown.

[e] The evidence of Kassell and Brand ('36-13) as to the presence of 0.7 per cent methionine in pure crystalline insulin is in conflict with the results of preceding investigators. Freudenberg, Dirscherl and Eyer ('30-1) obtained up to 0.9 per cent of methyl (CH_3-) and the equivalent amount of sulfur on treating amorphous insulin with hydroiodic acid according to the method of Kirpal and Bühn ('15-1), but crystalline insulin yielded neither methyl nor sulfur. This was confirmed by Jensen, Evans, Pennington and Schock ('36-12) who found the test for thiolmethyl negative on crystalline insulin when applied either before or after hydrolysis of the hormon. Miller and du Vigneaud ('37-15) showed that 100 ± 3 per cent of the total sulfur of insulin can be accounted for as cystine, but they admit that the accuracy of their determination does not include the presence of a small amount of methionine of the order of magnitude obtained by Kassell and Brand ('36-13).

Baernstein[4] previously (p. 345) referred to. The data have been condensed in Table III. A few data which are not of quantitative value except in as much as they represent minimum figures have also been incorporated (cf. superscripts [e] and [d] in the footnote to Table III). In addition there are the older investigations of Osborne ('02-1) and Mörner ('14-1) which in the light of present knowledge (cf. p. 337) suggest the presence of varying amounts of methionine in a number of proteins. These are (omitting those on which more definite knowl-

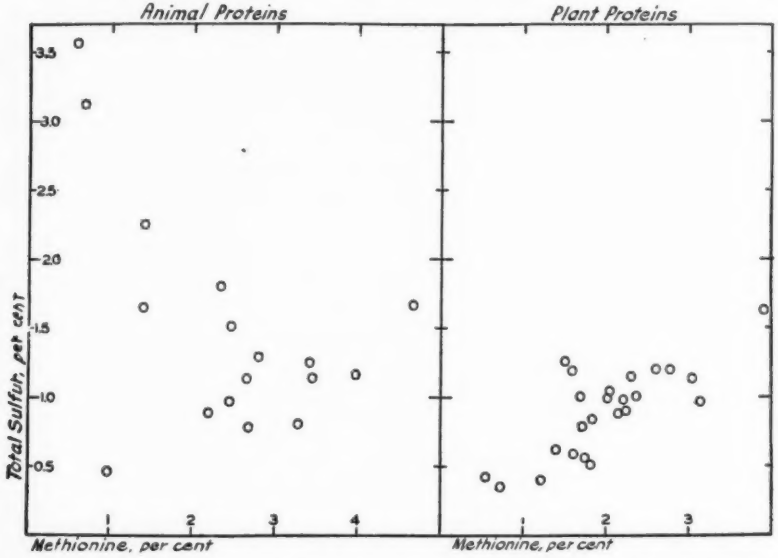


FIG. 1. The relation between total sulfur content and methionine content. The data used for this chart have been taken from Table III. Only those proteins on which both total sulfur and methionine content are available have been entered, and in those cases where different values have been given for the same protein (edestin hemp, zein, ovalbumin, wool, casein etc.) only the mean value has been plotted. The data on myosins from different genera have also been combined into one value.

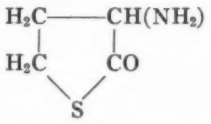
edge has become available and which are included in Table III): the plant proteins amandin (from seeds of almond and peach), excelsin (brazil nut), legumin, phaseolin (kidney bean), vicilin (globulin of legumes) and vigñin (cow pea), and the animal proteins globin, oxyhemoglobin, seralbumin and seroglobulin in the paper of Osborne ('02-1) and hemoglobin and seralbumin in that of Mörner ('14-1).

The foregoing distribution data taken as a whole are presumptive evidence for the conclusion that methionine may be an obligate constituent of the protein molecule, in as much as no protein is known that is free of sulfur, and since among the various proteins that have been examined for methionine none has

[4] Minor modifications have been suggested by Kassell ('37-10), Plimmer and Lowndes ('37-16) and Bailey ('37-1, '37-2).

been definitely found to be free of it. The list of plant proteins shows an average methionine content of approximately 2.0 per cent representing about 50 per cent of the total sulfur, the individual values varying between 0.5 per cent for arachin and 3.9 per cent for sativin, while the methionine share of the total sulfur varies between 28 and 76 per cent. The remaining sulfur, in plant as well as animal proteins, is chiefly present in the form of cystine; while a small part —about ten per cent in the average—of the total sulfur has not been definitely accounted for as either cystine or methionine sulfur. The methionine content of animal proteins tends to be somewhat higher in the average (2.6 per cent) than that of the plant proteins; but the methionine fraction of the total sulfur is about 50 per cent in the average as in the plant proteins, although it varies over a wider range (between 3 and 86 per cent). A plot, based on the data at present available, of total sulfur content against methionine content (Figure 1) suggests, for the animal proteins, that a high sulfur content tends to be associated with a low methionine content; cf. wool, insulin, ovomucoid. Among the plant proteins the opposite correlation is apparent: the highest methionine content is associated with the highest sulfur content (sativin) and low methionine content occurs together with low sulfur content (arachin, β-globulin of lima bean). Noteworthy may be the relatively high methionine content of the dynamic muscle proteins, which has become evident through the recent work of Bailey ('37-2), as compared with the high cystine content of the static keratins. The accumulating data increasingly indicate that methionine "is quantitatively the most important sulfur compound in an ordinary diet" ('32-10).

Normal Metabolism.—Studies dealing with fate and function of methionine in normal mammalian metabolism have been carried out on rat, rabbit, dog and man as the experimental subjects. Chase and Lewis ('33-3) found the rate of absorption of dl-methionine from the intestinal tract of white rats to be about 0.36 milli-equivalents per 100 gram of rat per hour as compared with the corresponding figure of 0.43 milli-equivalents for cystine. The absorption coefficient of glycine and alanine was found considerably larger, that of glutamic acid slightly larger, and that of leucine slightly smaller, than that of methionine. They also observed the presence of disulfide, as well as a test for –SH, in the urine after methionine feeding, both under conditions which never gave a positive reaction in normal urine. The observation that the sulfhydryl (–SH) reaction—with ammonia and nitroprusside—"attained its maximum intensity in 7 to 8 minutes" seems important as it is likely to indicate the presence of the thiolactone of homocysteine



in the urine.

This compound gives a slowly developing nitroprusside test ('34-1) while with free –SH the test appears instantaneously and fades away rapidly.

The effect of methionine on the fat metabolism of the liver of rats has been studied by Tucker and Eckstein ('37-21). Addition for 18 days, of 0.5 per cent of methionine to a diet containing 5 per cent casein and 40 per cent fat caused an approximately 40 per cent decrease in the lipid content of the liver, while addition of 0.5 per cent of cystine in place of methionine caused an increase of approximately 60 per cent. Previous observations of Channon and Wilkinson ('35-7) and Beeston and Channon ('36-5) that increased casein content of the diet lowered the lipid content of the liver while addition of cystine caused an increase, thus seem to find their explanation in an antagonistic relation between methionine and cystine, the effect of the former being the dominating one in casein in which the methionine-cystine ratio is unusually high. In this connection there should be mentioned the observation of Chase and Lewis ('33-3) that the glycogen content of the rat liver was unaffected by the feeding of dl-methionine, and the experiments of Vars ('33-11) on the phlorhizinized dog indicating that dl-methionine can be metabolized to yield glucose. The differential effects of added cystine in nitrogen metabolism, depending on the dietary protein fed ('37-12) may also be related to differences in the methionine content of the latter.

Experiments on rabbits by Virtue and Lewis ('34-16) and by du Vigneaud, Loring and Craft ('34-15), show that dl-methionine, either fed or injected subcutaneously, is readily metabolized, and indicate that its sulfur is chiefly converted to inorganic sulfate, the sulfur distribution in the urine being similar to that obtained when l-cystine is given, except for the fact that the increase of disulfide (−S−S−) in the urine is definitely more pronounced than following ingestion of cystine. A negative Sullivan test suggested that the urinary disulfide was not cystine but probably homocystine ('34-16). Similar results have been obtained on dogs. Pirie ('32-10) found that after ingestion of l-methionine, just as after that of l-cystine, about two-thirds of the sulfur given was excreted within two days as sulfate, while 11 to 16 per cent appeared as "neutral sulfur." Stekol and Schmidt ('33-9) found an increase in the creatinine output, together with increased production of inorganic sulfate and urea, when dl-methionine was fed to dogs maintained in nitrogenous balance. Stekol ('35-21) found that l- or dl-methionine, when fed to dogs maintained on a protein-free diet, was retained as readily as l-cystine and without causing a decrease in the output of urinary nitrogen. He concluded that "the retained l-cystine or either form of methionine sulfur is utilized. . . not necessarily in the form of protein, and that the adult dog possesses a store of sulfur other than, and in addition to the tissue protein." Andrews, Johnston and Andrews ('36-2) reported rapid absorption of dl-methionine from intestinal loops of dogs.

The only studies on the methionine metabolism of the normal human are those of Mueller ('23-2) and Medes ('37-14). After ingesting methionine the former found increased urinary excretion of free sulfate, while ethereal sulfate, neutral sulfur and total nitrogen remained unchanged. The latter found a rapid rise of sulfate excretion without increase in the excretion of total nitrogen, disulfide or the other sulfur fractions when either dl- or l-methionine was taken.

Practically 100 per cent of the sulfur ingested as methionine was excreted within 24 hours as inorganic sulfate, and the rate of excretion was much more rapid than that following ingestion of cystine, rather approaching the speedy sulfate excretion observed in the case of cysteine.

Growth.—The rôle of methionine in bacterial growth is of special interest as it was the search for a protein fraction necessary for growth of hemolytic streptococci, and which was removed from meat infusion by charcoal ('21-1), that led Mueller to the discovery of methionine ('23-1). Apparently however, methionine was not the only growth-essential factor absorbed by the charcoal as the purified compound did not possess the growth-inducing quality. On the other hand recent communications of Mueller ('35-16, '35-17) show that the development of diphtheria bacteria is enhanced in proportion to the amount of methionine added to the culture.

Hammett ('36-10) in his studies on developmental growth in which Obelia geniculata (family hydrozoa, phylus Coelenterata) serves as the experimental organism, found dl-methionine stimulative of new growth production and proliferation, the developmental phases of differentiation and organization being unaffected. The effective level as well as the toxic threshold were somewhat higher than in the case of compounds bearing a free $-SH$ group like cysteine.

The rôle of methionine in normal mammalian growth has been the object of a number of investigations which have culminated in important discoveries. Jackson and Block were the first to show that addition of dl-methionine to a cystine deficient diet will cause resumption of growth of young rats ('31-4). Their growth curves, obtained on a small number of individual animals, suggested that measured by the ensuing rate of gain in weight one sulfur equivalent of l-cystine is superior to two sulfur equivalents of dl-methionine and that the effect produced by cystine is not even exceeded when the amount of methionine sulfur is increased from two to four ('32-6). The growth-promoting action of dl-methionine was confirmed by Weichselbaum, Weichselbaum and Stewart ('32-15). They found, using the Sherman-Merrill ('25-2) cystine-deficient diet, that with equal sulfur equivalents the increase in the rate of growth produced by l-cystine was 1.7 times as large as that produced by dl-methionine. Later Jackson and Block ('33-4) compared the separated optical enantiomorphs, d- and l-methionine, and found that both stimulate the growth of cystine-deficient animals. Du Vigneaud, Dyer and Harmon ('33-12) found that addition of dl-methionine consistently produced somewhat more growth than that produced by one-half the amount of sulfur in the form of l-cystine. Comparison of these different results in their quantitative aspects is of limited validity on account of differences in the experimental conditions. Jackson and Block ('32-6) use a basic diet on which the weight of the animals remains approximately stationary, and their l-cystine supplement which serves as the standard of comparison is 1.2 grams per kilogram of the diet. The basic food mixture used by Weichselbaum *et al.* ('32-15) is one which induces considerable gain of weight by itself, in the absence of any added cystine or methionine, and their standard cystine ration is 2 grams per kilogram diet. Du

Vigneaud and associates ('33-12) employ a diet, which without supplement, produces rapid loss in weight, and their standard supplement consists of 3 grams of l-cystine per kilogram of food mixture. The essential result of this line of investigation seemed to be, first, that methionine can function in place of cystine in the promotion of normal growth, and second, that both d- and l-methionine are biologically available for this function, quite in sharp contrast to the case of cystine where it had been definitely established that only the naturally occurring l-compound has growth-promoting properties ('32-12, '33-7). However, the problem took on an entirely new aspect when in 1936 Rose and his coworkers ('36-18) communicated evidence showing that the dietary rôle of cystine in growth is definitely secondary to that of methionine. Their experiments ('37-17, '37-25) differ from all previous studies on mammalian growth in that the protein part of the food consists entirely of a balanced mixture of amino acids of defined constitution and purity. Under these conditions it appeared that on a diet which apparently satisfied all requirements of normal growth except those for organic sulfur, the animals could not be made to grow by the incorporation of l-cystine (6 grams per kilogram of food), while addition of the same amount of dl-methionine produced a normal rate of growth which could not be increased by increasing the amount of methionine given (to 14 grams) nor by giving 3 grams of l-cystine in addition to the 6 grams of dl-methionine. The authors conclude from their experiments—which also showed that d- and l-methionine are of equal effectiveness—that methionine is an essential dietary constituent, while cystine is, contrary to current conceptions, not an indispensable part of the diet. A confirmation of this conclusion is had in the report of Beach and White ('37-4, '37-24) that animals which are fed a diet in which arachin—a protein contained in peanuts and whose methionine content (0.5 per cent, '32-3) is exceptionally low— is the sole source of amino acids, do not respond with a gain in weight to the addition of l-cystine, but only to that of (dl-) methionine. This confirmation is also of value in that it discounts the possibility that the findings of Rose *et al.* may in some way be linked to the fact that their animals receive the nitrogenous part of their food not in the normal form of protein complexes but in the un-normal "predigested" form of free amino acid molecules. In order to reconcile the results of other investigators ('16-1, '25-2, '32-6, '33-12, '36-8, '37-5), demonstrating acceleration or induction of growth by addition of l-cystine and some of its derivatives to the diet, with the new picture it may be necessary to assume that in the tissue-building process l-cystine can be readily derived from methionine, while methionine, or at least a certain minimum of it, cannot be synthesized from cystine. Evidently the minimum non-replaceable methionine requirement—which alone is insufficient in amount even for maintenance of weight ('33-12, '36-8, '36-14, '37-5)—is supplied by the protein constituents (chiefly casein) of the basic diets that have been used.[5]

[5] Du Vigneaud, Dorfmann and Loring ('32-12) obtained a cystine-free casein hydrolysate by treatment with sliver ion (cf. '00-2, '30-2, '37-13). A diet containing 14 per cent of this material was sufficient for maintenance requirements and even partial growth ('33-12) while the animals responded with

A tentative picture of the quantitative aspects of methionine metabolism (of the albino rat) may be drawn as follows: Animals subsisting on a diet containing as little as 5 per cent casein respond vigorously to a cystine supplement ('33-12). Assuming a methionine content of casein of 3 per cent (cf. Table III) the diet contains 0.15 per cent of methionine. On the other hand, when the protein component of the diet is 15 per cent arachin, there is no response to cystine ('37-24).[6] The calculated methionine content of the diet, corresponding to 0.5 per cent methionine in arachin (cf. Table III) in this case is 0.075 per cent. Thus it appears that the minimum amount of methionine which cannot be replaced but can be supplemented by l-cystine is about 0.1 per cent (between 0.075 and 0.15 per cent). This is about one-sixth of the minimum amount of methionine (0.6 per cent) which in the absence of any cystine will insure a normal rate of growth ('37-17).[7]

Numerous questions arise from the new findings and remain to be investigated; such as the extent of functional equivalence between cystine and methionine, whether tissue l-cystine is actually produced from dietary methionine, and if it is equally well produced from d- and l-methionine. In view of the demonstrated similarity in the metabolism of methionine and cysteine in cystinuria (see below) which differs from that of cystine, the possibility has been suggested ('37-8) that in the growth supporting function methionine may be interchangeable with cysteine, even though not with cystine. However, the experiments of White and Beach ('37-24) indicate that neither cystine nor cysteine can substitute for the critical minimum requirement of methionine.

In the light of the newly disclosed primary position of methionine new importance is gained by the problems of the biological origin of such important tissue components as glutathione, insulin or keratin which are prominent by their high content of cystine or cysteine. That methionine contained in the protein of grasses may wholly or in part represent the source of the large amounts of cystine continuously elaborated in the wool fleece of sheep is a possibility which has been variously discussed ('32-8, '32-11, '33-5, '34-3). While it has been shown ('37-1) that in grass proteins one-half of the total sulfur may be present as methionine, the direct experimental evidence of Marston ('35-15) seems to indicate that if the sheep can convert l-methionine into keratinous cystine at all, this ability is very much smaller than that of utilizing cystine or cysteine.

Special Metabolic Aspects.—(a) Enzyme Systems.—With regard to the en-

vigorous growth to the addition of approximately 4 grams of l-cystine per kilogram of diet. Obviously the methionine content of the hydrolysate—presumably unaffected by the silver treatment—was responsible for the positive response to cystine.

[6] Apparently in the vegetable protein diets of Kotake *et al.* ('36-14), with a minimum sulfur content of 0.023 per cent (5 per cent legumin containing 0.46 per cent sulfur), the minimum methionine requirements are still satisfied, as growth response on addition of l-cystine (together with l-tryptophane) is shown.

[7] In this computation, very approximate at best, no allowance has been made for the variations in food intake, which in some investigations have been overcome by giving fixed amounts of the sulfur compounds, and sometimes also of the basic diet; instead of supplying *ad libitum* the food mixture which contains the sulfur compound in a definite ratio.

zyme chemistry of methionine there are a few observations on record in connection with studies on the general enzyme metabolism of amino acids. Krebs ('35-11) extracted an enzyme system from pig kidney which he found to be specific for the oxidative deamination of the non-naturally occurring amino acids, according to the scheme (d-) $R-CH(NH_2)-COOH+\frac{1}{2}O_2 \rightarrow R-CO-COOH+NH_3$. Of a series of amino acid studied by this system which he called d-aminoacid deaminase he found that measured by the oxygen uptake, methionine showed the highest rate of oxidation, being about twice as high as that of its isomer S−ethyl cysteine and about four times as high as that of cystine while the rates of other amino acids were five to fifty per cent of that of methionine. A similar preparation, presumably specific for d-amino acids, and obtained from rat kidneys was described by Bernheim and Bernheim ('35-2). dl-Methionine showed an oxygen uptake of one-half atom per molecule, and at a rate similar to that of other amino acids. Krebs ('35-11) also described another and more labile system specific for the deamination of the natural modifications and termed l-amino acid deaminase, but its action on methionine was not studied. However, Bernheim *et al.* ('35-3) showed evidence for the existence of a similar system, being more or less specific for the natural form of the amino acids, in "resting" (non-proliferating) Baccillus proteus vulgaris. The extent of oxygen uptake and ammonia liberation, corresponding to one-half the theoretical amounts for oxidative deamination when dl-methionine was used, indicated, by analogy with other amino acids studied that only the natural form reacts. It is noteworthy that while the natural forms of leucine, phenylalanine and, presumably, methionine consumed 1 atom of oxygen, other amino acids used higher amounts, for instance with serine, alanine and proline the maximum uptake was 3, 4 and 7 atoms respectively, indicating a more extensive oxidative break-down. In studies on heart tissue on the other hand, ('37-3) among some 12 amino acids studied only l-cystine and l-proline were found to undergo oxidative deamination, while (dl-) methionine was among the amino acids that were not susceptible to the deaminase system present. In connection with bacterial action mention must be made of the paper of Akobe ('36-1) which reports that l-methionine is converted into the d-form of its hydroxyanalog, α-hydroxy-γ-methiobutyric acid by B. oidium lactis, while by Bacillus subtilis it is turned into the α-form of the same compound. Woods and Clifton ('37-26) report that, among other natural amino acids l-methionine is decomposed with formation of carbon dioxide, ammonia and hydrogen by an obligate anaerobe, Bacillus closteridium tetanomorphum.

By comparison with the enzyme systems of oxidative and hydrolytic deamination, which attack the non-specific part of the amino acid molecule, little is known about any enzyme systems concerned with the oxidative desulfuration which according to the metabolic studies must be important factors acting with high specificity on the different types of sulfur bearing amino acids. Pirie ('34-10) studied sulfate formation *in vitro* and demonstrated that l-methionine, as well as cysteine but not cystine, yields sulfate ions in the presence of slices of rat liver and rat kidney, and that also a faint test for free −SH groups is

produced. Tarr ('33-10, '34-11) found an enzyme system to be present in certain heterotrophic bacteria (*P. vulgaris* and *S. Marcescens*) which produces hydrogen sulfide from organic sulfur compounds and which seems to be specific for molecules which combine a sulfhydryl or disulfide group with an amino group since in the case of cysteine and cystine about 80 per cent of the sulfur was converted to hydrogen sulfide. Methionine and cysteine derivatives of an analogous structure like S—ethyl and S—benzyl cysteine gave no hydrogen sulfide while homocystine, the disulfide corresponding to methionine, did so to the extent of 12 to 24 per cent. Chaix and Fromageot ('36-7) state that dl-methionine is similar to cystine, reduced and oxidized glutathione, thiolactic and thioglycollic acid in its stimulating action on the fermentation of glucose by propionic bacteria.

(b) Taurocholic Acid Formation.—The possibility that dl-methionine may serve as a source of the sulfur (taurine) component of taurocholic acid is left open by a report of White ('36-23). White caused discontinuance of the growth of young rats by addition of cholic acid to their diet, presumably because hereby the amount of cystine available for tissue-building is reduced, as it combines with cholic acid in the formation of taurocholic acid. Prompt resumption of normal growth could be induced not only by adding l-cystine to the diet (3.6 grams per kilogram) but also and with equal effectiveness by the equivalent amount (4.5 grams) of dl-methionine. However, the author leaves the question open as to whether dl-methionine actually enters into the formation of taurocholic acid or whether it acts indirectly in the elimination of the excessive cholic acid by some other detoxication mechanism.

(c) Blood Coagulation.—Sterner and Medes ('36-20, '36-21) studying the effect of l-cystine, dl-methionine and some other substances on the coagulation of blood made the interesting discovery that while *in vivo* both cysteine and methionine delay the coagulation, *in vitro* of the two compounds only cysteine had such an effect—which was proportional to the amount added—while methionine even when its concentration was increased to 1 per cent remained without influence. *In vivo*, after injection or ingestion of the compounds, methionine displayed its maximum effect 2 to 4 hours after it was given, as compared with less than one hour in the case of cysteine.

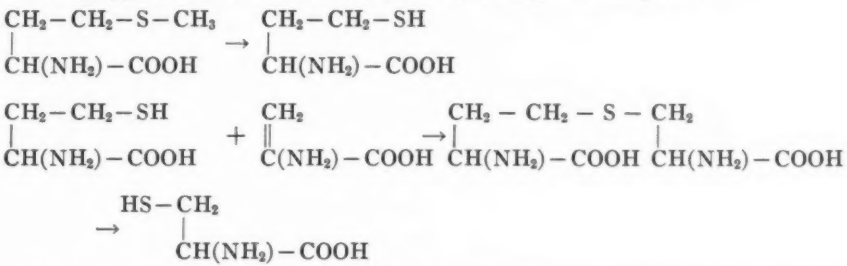
(d) Detoxication.—The excretion of bromo-phenylmercapturic acid after feeding of bromobenzene has been a frequent object of study as an example of the detoxifying action of cystine. The excreted detoxication product is a S-bromophenyl, N-acetyl derivative of cysteine, and endogenous or dietary cystine has been shown to play a rôle in its formation (for a discussion of the literature, cf. '32-16). White and Lewis ('32-16) found that when dl-methionine was given to a dog which, being fed a diet of low sulfur content, simultaneously received bromobenzene the excretory picture in terms of nitrogen output and sulfur partition was similar to that resulting when cystine was given in place of methionine. A similar process of detoxication was indicated in both cases by similar decreases in nitrogen catabolism and increases in the output of "neutral sulfur." White and Jackson ('35-24) also showed a functioning of dl-meth-

ionine similar to that of l-cystine in connection with bromobenzene poisoning. In their experiments the growth of young rats which was caused to cease by the administration of bromobenzene was fully reestablished when equivalent amounts of l-cystine (1.2 grams per kilogram of diet) or dl-methionine (1.5 grams) were given in addition to the bromobenzene. Stekol ('37-19) showed the same phenomenon of reestablished growth by either l-cystine or dl-methionine when the poisoning agent was naphthalene instead of bromobenzene. The experimental picture in the growth studies with either bromobenzene or naphthalene is similar to that obtained by White ('36-23) in connection with poisoning by cholic acid (cf. Taurocholic Acid Formation). In all these cases the detoxifying action seems to be specific for cystine or methionine since sulfur in other forms, such as taurine or inorganic sulfur or sulfate, as well as other amino acids were found ineffective. In another phase of the detoxifying action of the organism which finds its expression in increased excretion of ethereal sulfates dl-methionine was also found effective. Stekol ('36-19) found an increased output of ethereal sulfates as a result of feeding isobarbituric acid to dog or pig when dl-methionine was given simultaneously. The participation of dietary sulfur in the formation of ethereal sulfates is less compound specific however, as not only l-cystine or cysteine but also sodium sulfate produced the same effect as dl-methionine.

It should be mentioned here in connection with the animal organism's mechanism of detoxication involving sulfur compounds, especially as regards the equivalent action of cystine and methionine, that Stekol ('37-20) in a series of studies has presented evidence in support of his conclusion that the sulfur involved is of endogenous origin and that the favorable effect of an increased supply of such compounds as cystine or methionine is a secondary one in that it serves for rapid reconstruction of tissue proteins depleted of its organic sulfur constituents. Brand ('33-2, '34-5) has called attention to the hypothesis of Waelsch ('30-3) which considers glutathione as the active agent concerned in detoxication, and discussed the possible rôle of methionine as a source of the cysteine component of glutathione.

(e) Cystinuria.—Interesting and important contributions to the problem of methionine metabolism have arisen from studies concerned with the metabolic defect known as cystinuria. In 1934 Brand and his associates ('34-5, '34-6, '34-7, '35-6) announced their findings showing that in the cystinuric human —whose chief symptom is excretion of cystine in the urine or even deposition of cystine stones in the bladder—addition of cystine to the diet (of low protein content) does not cause an increase in the excretion of urinary cystine, but rather an increase in the excretion of inorganic sulfate, indicating ready oxidation of the cystine fed. The result was similar when glutathione was fed but fundamentally different in the cases of either cysteine or dl-methionine. The total increase in the sulfur excretion was less than in the case of cystine feeding, but it was largely due to increased cystine excretion, the latter accounting for about one-third of the sulfur fed in the form of cysteine or methionine. The apparently paradoxical result that methionine ingestion produces excretion of

cystine, while ingestion of cystine does not, has been confirmed when not the free amino acids were fed but proteins differing widely in their methionine-cystine ratio ('37-8). When casein which contains about 14 times (molecular ratio) as much methionine as cystine, was fed on the one hand, and lactalbumin, in which the corresponding ratio is only 1.4, on the other the excretory picture was in harmony with the expectation derived from the results obtained with methionine and cystine when fed as the free amino acids. Lewis *et al.* ('36-15) on their part obtained results on a cystinuric individual which agree with those of Brand and associates as regards the relative effects of the ingestion of cystine, cysteine or methionine on the urinary output of sulfur compounds. When the basic diet was of higher protein content the amount of extra cystine excreted after the feeding of methionine was less than when the protein content of the diet was low. On the other hand, Andrews and Randall ('35-1) in studies carried out on a cystinuric boy found oxidation to sulfate to the extent of 60–80 per cent and no significant increase in urinary cystine when dl-methionine was fed. It seems that until more than a few single cases of cystinurics have been studied the possibility of variations in the metabolic mechanisms of different individuals must be considered. On the basis of their findings Brand and associates have concluded that the metabolism of cystine and cysteine proceeds along different pathways—a conclusion that is supported by the results of Medes ('37-14) on the normal human—and that *in vivo* methionine can be converted into cysteine. For this conversion they suggest ('36-6) as a working hypothesis for further study the following reaction path:



(f) Miscellaneous Pathological (Deficiency Disease, Fatty Liver, Tumor Growth).—In connection with the problem of the biological relations between methionine and cystine an observation of Weichselbaum ('35-23) seems of great interest and may deserve further study. This author found that young albino rats when kept on the Sherman-Merrill cystine deficient diet ('25-2) for more than five weeks in a large proportion of cases developed a characteristic syndrome with fatal ending. The addition of l-cystine or dl-methionine to the diet prevented the appearance of this deficiency disease, but once the symptoms had definitely appeared only cystine brought about recovery (in 18 cases out of 25 treated) while methionine remained without effect (in all of 17 cases treated). The appearance of the disease was observed in three separate batches of Wistar strain rats obtained from two different sources, and males were found

more susceptible than females: 80 to 90 per cent of the males became ill, compared with 30 to 50 per cent of the females. It is interesting that other authors who have used the Sherman-Merrill and other deficiency diets (cf. section on Growth) apparently have not encountered the syndrome of Weichselbaum although animals have been maintained in sulfur deficient diets for much longer periods than 5 weeks; e.g. 230 ('25-2), 90 ('32-6), 70 ('32-12), 60 ('33-12) or 40 ('33-7) days. The phenomenon may bear a relation to the observations of Tucker and Eckstein ('37-21) on the effect of supplementary methionine and cystine on the production of fatty livers by adult rats. Addition to a diet containing 5 per cent casein (corresponding to about 0.15 grams cystine and 1.5 grams methionine per kilogram diet) of 5 grams of cystine or methionine (per kilogram diet) caused within 2½ weeks very definite changes in the lipid content of the liver. The liver fat content of the animals receiving the cystine supplement was about 60 per cent higher and of those receiving methionine about 40 per cent lower than that of the controls. These results are in agreement with and explanatory of previous findings by Channon *et al.* ('35-7, '36-5) on relations between the production of fatty livers and increases in the supply of dietary casein or cystine. Chase and Lewis ('33-3) showed that the glycogen content of the liver of rats is not affected by the feeding of dl-methionine.

Regarding the possible rôle of methionine in the metabolism of malignant growth the only study seems to be that of Lustig and Wachtel ('35-14) in which it is reported that dl-methionine was found without effect on the growth of tumor cells *in vitro* or *in vivo*, contrary to cysteine and cystine both of which showed a definite anticarcinogenic action expressed by a smaller number of takes, slower tumor growth and longer life after inocculation of mice with tumor tissue.

Relative Biological Position of d- and l-Methionine and of Some Related Compounds.—The question of the biological equivalence of the two optical isomers of methionine is of interest on general biochemical grounds, but especially in connection with the problem of the functional interchangeability of methionine and cystine. Evidence that the unnatural isomer of methionine is metabolized in addition to the natural one ('33-9), that both stimulate growth when added to a cystine deficient diet ('33-4), that both are of equal ('37-25) or almost equal ('37-17) efficiency in reestablishing the growth of animals kept on a diet devoid of sulfur-containing amino acids,—appears in sharp contrast with the situation of cystine where only the natural isomer can be utilized for growth purposes ('32-12, '33-7) and where the unnatural form seems to offer notable resistance to the normal oxidative metabolic breakdown ('34-13). On the other hand there are also indications that not under all circumstances and in all biological mechanisms both forms of methionine react identically: in normal human metabolism the l-compound was distinctly found to be somewhat more rapidly oxidized than the dl-form ('37-14) and there is evidence for the presence, in kidney tissue and some bacteria, of enzyme systems that act selectively on either the d- or the l-form of amino acids, including methionine (cf. section on Enzyme Systems).

A number of close chemical relatives of methionine have been studied biologically,[8] and some insight has been gained into the chemical problems of methionine metabolism as a result of such studies. S-Methylcysteine, the next lower homolog of methione, was found to be toxic to the dog and to be metabolically oxidized only to a limited extent ('32-10). In the rabbit, however, it was found to be oxidized to the same extent as methionine and no toxic effects were observed ('34-15). In the normal human the compound was metabolized in a similar manner ('37-7). However, excretion of about one-third of the sulfur content as "neutral sulfur," presumably in the form of the unchanged substance ('37-7) contrasts with the behavior of methionine the sulfur of which in the human is practically completely excreted as sulfate ('37-14). S-Methylcysteine definitely differs from methionine in being incapable of reestablishing stunted growth of rats caused by a cystine-deficient diet ('32-4) and in not being demethylated with formation of reactive $-SH$ by kidney slices ('37-7). In a cystinuric patient the compound was much less readily metabolized than methionine or cysteine ('37-7).

S$-$Ethylcysteine, while much less toxic than the methyl compound, was not converted to sulfate by the dog ('32-10). It also was incapable of being utilized for growth purposes ('32-4), as was homomethionine, the next higher homolog of methionine ('35-9). Homocystine, however, the product of demethylation of methionine, was found to be capable of being thus utilized ('33-12), both optical forms being practically equally effective in replacing an equimolar amount of l-cystine ('35-10). There are however, data suggesting that the biological oxidizability of the d-compound is slightly less than that of the natural isomer ('37-23), just as there are indications for a similar difference in degree for the two forms of methionine ('37-14). While the compound thus behaves very similar to methionine, even to the extent of being able to substitute for the minimum requirements of methionine which cannot be replaced by cystine ('37-24) it is interesting that in the cystinuric organism the extent and mode of its metabolism was found unlike that of methionine and very similar to the behavior of cystine ('34-7, '35-5). The cystinuric metabolism of its reduced form, homocysteine, was comparable with that of methionine in that it led to increased disulfide excretion the major part of which consisted of cystine, the balance probably being the form of homocystine ('35-5). The metabolism of homocystine in the normal rabbit has been found almost identical with that of methionine ('34-15). Unlike homocystine, the disulfide corresponding to methionine, the next higher disulfide which has been called pentocystine was found to be devoid of growth-supporting properties ('35-9). It has been stated that the sulfoxide of methionine, unlike those of S$-$ethyl or S$-$benzylcysteine, is as readily oxidized by the organism of the dog as methionine itself, but the compounds have not been described chemically ('32-10).

In connection with the metabolism of methionine some consideration has been given to the effect of substitutions in the amino group. The optically in-

[8] For structure and preparation cf. section on Salts and Other Derivatives.

active forms of N-methyl methionine and the corresponding disulfide di-N-methyl homocystine were both found to produce about the same growth effect as l-cystine when the amount fed corresponded to twice the sulfur content of the latter ('36-17). It cannot be definitely inferred from the experiment whether or not only the naturally occurring moiety of the racemate is effective. However, this has been reported for the N-formyl derivatives of methionine, where the levorotatory form was found to be equivalent to l- or d-methionine on a cystine-deficient diet, while the d-compound was ineffective ('33-4), and for the N-acetyl derivatives of homocystine ('37-23). While both l- and d-homocystine are equally effective in stimulating growth ('35-10) upon acetylation this capacity is lost in the case of the d-compound, while in the case of the naturally occurring l- form it is retained undiminished ('37-23). Similarly physiological oxidizability (conversion to inorganic sulfate) was found greatly decreased by the acetylation of the d-compound while the oxidizability of diacetyl l-homocystine appeared equal to that of the parent substance ('37-23).

Contrary to acetylation benzoylation apparently completely blocks physiological availability. After feeding of (dl-) N-benzoyl methionine to the rabbit the compound has been reisolated from the urine and absence of a disulfide reaction and of increased sulfate excretion indicated that neither oxidation nor demethylation had taken place ('33-13, '34-16).

While thus substitutions in the amino group definitely affect the growth supporting properties of the methionine molecule it has been reported that α-hydroxy-γ-methiobutyric acid, the compound resulting from replacing the amino group of methionine by hydroxyl, can be utilized for growth purposes ('32-4) equally in both its dextrorotatory and levorotatory form ('36-1). In the cystinuric the compound is, contrary to methionine, only partly oxidized to sulfate, while a part appears in the form of excreted cystine and the remainder probably is excreted as the unchanged compound ('37-6). In the normal human the compound was very readily, if somewhat less so than methionine itself, converted to sulfate ('37-14), differing strongly from the behavior of the corresponding dihydroxy analog of cystine which was excreted slowly, and apparently chiefly in unchanged form ('37-14).

CONCLUSION

An examination of the methionine literature shows numerous lines of departure, both biological and chemical, waiting to be investigated. Some of the problems which need further consideration, and the progress of which is likely to further emphasize the rôle of methionine as one of the leading factors in the metabolism of sulfur, are: the physical constants of the compound, the chemical mechanisms involved in the isolation, the difficulties of quantitative conversion of its sulfur to sulfate in analytical procedures contrasted by the ease of this conversion in normal metabolism, the nature of the equilibria involving methionine, the thiolactone of homocysteine and homocysteine itself, the metabolic rôle of the latter two compounds, the biological rôle of methionine as a potential source of thiol groups, its dietary importance, its rôle in cystin-

uria as a source of cystine and the extent of the apparent interchangeability of the dextro and levorotatory forms.

The endeavor has been to make the present review reasonably complete up to and including the literature of the year 1937. Acknowledgement is made of the help derived from previous reviews dealing with the biological chemistry of sulfur compounds ('24-1, '31-5, '32-7, '33-6, '35-12, '35-13, '36-22, '37-22).

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PERIODICITY IN THE GROWTH OF THE ORANGE TREE

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The relations between radial growth of the cambium and elongation of shoots and roots have been briefly investigated in six-year-old Navel orange trees at the Citrus Experiment Station at Riverside, California.

Periodicity in the growth of shoots is characteristic of the growth of citrus trees in the region mentioned (Reed, '28, Reed & Halma, '26) and is related to other changes in the physiology of the tree.

Mid-seasonal pauses in elongation and in cambium activity in the stems and roots of woody plants may generally be attributed to the effects of external factors, such as temperature and moisture, rarely to depletion of the food supply. Accumulated observations have made possible some correlations between the growth of terminal buds and the activity of cambium in deciduous trees and shrubs, but the relations between the two is not so clear in evergreens, or in trees which carry at least one seasonal suite of leaves at all times. Nor has any definite connection yet been established between the seasonal or periodic action of roots and trunks, beyond the fact that the larger basal parts of roots usually grow in diameter at the same time as the basal part of the trunk. In Arizona, periodicity of root growth is well marked in *Citrus aurantium*, *Vitis vinifera*, *Prosopis velutina*, and *Parkinsonia torreyana* (Crider, '28). About the first of December, the roots cease growth, but resume activity the latter part of March.

The orange trees from which these records were obtained in 1935 were propagated from selected buds of the variety Washington Navel, budded on sweet orange root stocks. This combination of scion and stock gives trees in which there is a normal fusion of tissues at the bud union (Webber, '26) since both belong to the same species. The trees ranged from six to eight feet in height at the time the observations were made.

The subjoined observations make it evident that periodicity in the growth of roots and trunks of these orange trees, as described below, cannot be ascribed to environic factors, to varying food supply, or to depletion of reserve food materials. The monthly mean maximum air temperatures rose from 19.4° C in March to 36.1° in August and declined to 27.3° in October. The mean monthly maximum soil temperatures, at a depth of 30 cm., rose from 15.6° C in March, to 32.2° in August and declined to 26.1° in October. Throughout the dry season the soil in which these trees grew was regularly and carefully irrigated in accordance with good orchard management. The radial growth of the tree trunks was measured by a dendrometer appropriately placed 25 to 30 cm. above the bud union. Changes in volume were recorded by the movement of a pointer which magnified the enlargement 15 times. The rhythms of elongation of shoots

and roots were determined by inspection without making quantitative records.

The observations were made simultaneously on four trees. The dendrometers were installed January 23, 1935, and frequently inspected. Figure 1 shows the cycles of cambial growth, shoot growth, and root growth for a representative tree.

Two of the trees showed cambial activity the last of March, but there was no appreciable enlargement until early in April although the first cycle of shoot growth started early in March.

The increments of radial growth in the first cycle reached a maximum the first of June and then decreased until early July. The second cycle started in

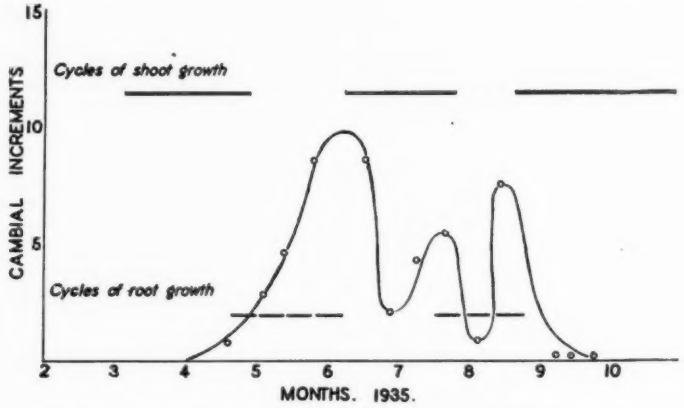

FIG. 1. Diagrammatic representation of the growth activity of a six-year old orange tree. The three-modal curve represents the cambial increments as registered on the dendrometer at 10-day intervals. The solid lines (above) represent the duration of the cycles of shoot growth; the broken lines (below) similarly represent cycles of root growth.

early July and stopped in early August and produced less enlargement than the first. The third and last cycle in three of the four trees started early in August and ended rather indefinitely in September. The greatest radial increments of the trunks were produced by the activity of the first cycle.

It is highly important to note that the first cycle of cambial growth followed the first cycle of growth of shoots. This sequence occurs in other evergreen trees, e.g. *Quercus agrifolia*, in which there is no cambial growth at the beginning of a season until after a new suite of leaves appear (MacDougal, '36) and flowers mature. It seems logical to assume that cambial growth in such cases depends, not on a supply of plastic food materials, but upon auxin produced by elongating shoots.

The cycles of shoot elongation and cambial growth showed a strong tendency to alternate, as shown in Figure 1. The first cycle of shoot growth was probably made at the expense of materials stored in the previous season. The greatest

growth of elongation of shoots occurred early in the spring and ceased before any distinct enlargement of trunks was recorded by the dendrometers. The second cycle of shoot growth started when the first cycle of cambial growth was at its maximum in three of the four trees. In the fourth tree, the first cycle of cambial growth did not reach its maximum until mid-July, consequently it was synchronous with the second cycle of shoot elongation. The third cycle in shoot growth in all of the trees was principally produced after the third cycle of cambial growth had ceased. The trees were in blossom during the latter part of March and early April, subsequently the fruits were developing, but apparently the growth of the trees was not controlled by the processes involved in fruit development.

The first cycle of root elongation, which was determined by observing the progress of formation of white terminals on the brown fibrous roots, coincided rather closely with the first cycle of cambial activity, but the second was only approximately synchronous with the second and third cycles of cambial activity. There were but two distinct cycles of root elongation in the period March 1 to October 1, when it was necessary to terminate the observations. The first cycle began late in March and ceased late in June; the second started in July and ended late in August. The initiation of root growth was probably influenced to some extent by soil temperature, but the cessation in summer was certainly not due to low temperatures. It seems impossible at present to explain the cyclic growth of roots on any simple basis, although it was probably affected by temperature and hormonic communication between root and shoot.

When the orange root has once started to grow, there is a temperature gradient. The maximum growth of young orange seedling roots in nutrient solution is between 24° and 27° C (Girton, '27) determined by measurements made on seedlings maintained two weeks at controlled temperatures.

The observations show that discontinuous shoot growth extended over a a period of nearly eight months, cambium growth five months and root elongation a total of about three months.

The observations may be summarized by saying that the cyclic growth of these orange trees gives important evidence of correlations in activity of meristems of shoots, cambium, and roots. In addition to the effects of various physical factors, there were inherent physiological factors affecting the activity of meristematic cells and groups of cells.

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STUDIES ON THE TRANSPLANTABILITY OF INDUCED AND SPONTANEOUS TUMORS OCCURRING IN MICE OF PURE INBRED STRAINS[1]

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A study of the transplantability of sarcomata induced by dibenzanthracene and of spontaneous mammary-gland carcinomata has been carried on in this laboratory for the past three years using certain pure inbred strains of mice, designated respectively as A (albino), C_3H (black agouti), BA (Bagg albino), and C_{57} (black).[2] This study has shown that, with few exceptions, tumors arising in mice of a pure inbred strain are specific for that strain.

The sarcomata were induced by an injection of 0.8 mg. of 1:2:5:6-dibenzanthracene dissolved in lard or olive oil into the right axilla of the mice. This caused an acute inflammatory reaction accompanied by the formation, within 48 hours, of a cyst around the injected material. The inflammatory reaction continued for some time but later subsided. The cysts became attached to the muscles of the chest and remained quiescent for varying periods of time before a tumor began to develop.

In sections of cysts made during this time an occasional cell was seen that appeared to have become altered, as described by Lewis (1936) while in sections of cysts made later usually about 90–100 days after the injection these changed cells were found to be in active multiplication and from that time on the tumor developed rapidly.

Andervont (1934, 1935, 1936) has studied the proportion of mice that develop tumors at the site of the injection of dibenzanthracene and the transplantability of these tumors in a number of strains. In our experiments it is probable that every mouse injected with dibenzanthracene would have developed a tumor had it lived long enough, for while the majority of tumors appeared within 90–150 days after the injection in some instances mice that had failed to develop tumors within six to eight months, and so were considered negative, 12–15 months later developed tumors at the injection site.

Transplantation of the cysts did not hasten the development of the tumors. Cysts transplanted earlier than 60 days after the injection of dibenzanthracene into mice of the same strain failed to grow into tumors, while others transplanted later than that did so. The results obtained on the later transplantations were as follows: Cysts from 4 C_3H strain mice, transplanted into mice of

[1] Aided by a grant made to Dr. Warren H. Lewis from the International Cancer Research Foundation.

[2] The mice of the A and of the C_3H strains were purchased from the Roscoe B. Jackson Memorial Laboratory. The mice of the BA strain, originally obtained from Dr. E. C. MacDowell of Cold Spring Harbor, and those of the C_{57} strain, originally obtained from the Roscoe B. Jackson Memorial Laboratory, were bred in our laboratory.

the same strain 87 to 122 days after the dibenzanthracene injection, resulted in 4 tumors, and these were successfully passed into a second generation 31 to 51 days later; a hematomatous cyst from a C_3H strain mouse, transplanted 96 days after injection, resulted in a tumor that was passed 37 days later; and cysts from two C_{57} strain mice, transplanted into C_{57} mice 145 and 161 days after injection, resulted in two tumors that were passed into a second generation 35 and 39 days later. Cysts from 2 BA strain mice, transplanted into BA mice 133 days after injection, resulted in two tumors that were passed into a second generation 67 and 74 days later. On the other hand, cysts from 11 C_{57} strain mice, transplanted 125 to 189 days after injection, and others from 4 BA strain mice, transplated 133 to 144 days after injection, failed to develop into tumors.

STRAIGHT-LINE TUMORS

The induced sarcomata were numbered from 1 to 200 in the order of their occurrence. They were passed through many generations by transplanting a fragment of the tumor from one mouse into the right and left axilla of two other young mice (6 to 10 weeks old) of the same strain. Tumors transplanted in this way are called the straight-line tumors. Tumor tissue used for experimental purposes was taken from a straight-line tumor at the time it was passed to another generation. If by any chance the two mice bearing a straight-line tumor died, that particular tumor was lost, as no experimental tumor was substituted for a straight-line tumor. The straight-line tumors proved to be 100 per cent transplantable in mice of the same strain.

RATE OF GROWTH

The rate of growth and the size attained by the transplants of straight-line tumors were about the same in the majority of the transplantations into young mice. A few grew more slowly (18 to 21 days) and a few more rapidly (9 to 12 days), but the majority required 13 to 16 days to reach the size of tumor used for transplantation purposes. The rate of growth could be regulated to some extent by the size of the piece transplanted. Tiny fragments of tumor introduced, one into the right and one into the left axilla grew into tumors which brought about the death of the host in 54 days; while larger pieces, the size of eight fragments, introduced by means of a 16-gauge trochar (our usual technic) brought about the death of the host in 26 days.

STRAIN-SPECIFIC TUMORS

Owing to the scarcity of mice, only 123 of the dibenzanthracene-induced tumors were inoculated into mice of other strains than that in which the tumor originated; 114 of these proved to be strain specific; that is, in no instance did a graft of one of these tumors grow progressively in a mouse of any of the pure inbred strains inoculated except that in which the particular tumor originated.

REGRESSING TUMORS

Transplants of every one of the tumors exhibited some growth in the first 4 or 5 days after inoculation into a host of an alien strain, but, except in the few

instances described under non-strain specific tumors, this growth later regressed. The transplants of the majority of the tumors did not grow larger than a small pea; a few reached the size of a lima bean, while those of four of the tumors grew quite large (20×10×10 mm.) in some, not all, of the inoculated mice of certain alien strains. The growths remained stationary for several days and then regressed. Since regression of growth of implants made into mice of the strain in which the tumor originated did not occur, the growths of these four tumors in alien strain mice furnished our only material for studies on regression.

NON-STRAIN SPECIFIC TUMORS

Nine of the straight-line tumors grew progressively when transplanted into a mouse of one or more of the alien strains. When the tumors arising in these hosts were transplanted into mice of the A, BA, C_3H and C_{57} strains, some of them grew only in mice of the strain in which the tumor originated, while a few grew again in some of the mice of other strains.

EXPERIMENTALLY OBTAINED NON-STRAIN SPECIFIC TUMORS

In some investigations on immunity a number of mice of one strain were repeatedly inoculated with pieces of straight-line tumors of an alien strain to the growth of which they had previously proved refractory. This procedure resulted in breaking down the resistance of a number of the treated mice permitting the growth of some of the grafted tumors and also in changing some of the tumors so that they were thereafter transplantable into mice of other strains. Ten series of experiments on repeated inoculations were carried out.[3] The results briefly were as follows:[4]

SERIES I. Implants of 12 of the tumors that originated in strain A mice were repeatedly introduced into 24 mice of the BA strain. Fifty-four tumors resulted 1 No. 6, 1 No. 7, 18 No. 9, 2 No. 11, 9 No. 14 and 23 No. 20), and when these were transplanted into normal mice of alien strains it was found that 3 of the tumors (No. 9, No. 14 and No. 20), previously transplantable only in mice of the A strain, had become so changed that they were transplantable into mice of other strains.

SERIES II. Implants of 6 BA strain tumors were repeatedly introduced into 20 mice of the C_3H strain. Thirty-two tumors (8 No. 1, 6 No. 3, 17 No. 6 and 1 No. 9) resulted. Tumors No. 1 and No. 3 were passed through two serial transplantations in mice of C_3H strain, but only No. 6 was passed through 5 generations in mice of other strains.

SERIES III. Implants of 6 C_3H strain tumors were repeatedly inoculated into 8 mice of the BA strain; 11 tumors (7 No. 11 and 4 No. 4) resulted. Tumors No. 4 and No. 11 were transplanted through 5 generations in mice of other strains.

SERIES IV. Implants of 6 C_3H strain tumors (No. 11 was not included among them) were repeatedly inoculated into 12 mice of the BA strain; 9 tumors (1 No. 4,

[3] The first eight series of experiments have been described in previous publications (Lewis and Lichtenstein, 1936, and M. R. Lewis, 1937).

[4] An effort was made to transplant every one of the tumors that grew in the repeatedly inoculated hosts through five or more generations in mice of the four strains. This proved to be impossible, partly because of insufficient normal young mice and partly because of the untimely death of some of the tumor-bearing animals.

1 No. 8, 1 No. 13 and 6 No. 25) resulted. Tumors No. 4 and No. 25 were transplanted through 5 generations in mice of other strains.

SERIES V. In this series 24 BA strain mice were repeatedly grafted with one type of tumor from C_3H strain; 6 mice received tumor No. 4, 6 tumor No. 8, 6 tumor No. 12 and 6 tumor No. 14. Only 3 tumors resulted, all of them No. 4, and these proved to be thereafter transplantable into mice of other strains.

SERIES VI. 11 BA strain tumors were repeatedly inoculated into 16 mice of the C_{57} strain. No tumors resulted. After a lapse of 23 days these mice were again repeatedly inoculated with 3 BA strain tumors not included among the first 11. Twenty-six tumors resulted; 16 of these were No. 35 and 10 were No. 48. Tumors Nos. 35 and 48 were transplanted through 5 generations in mice of other strains.

SERIES VII. Implants of 6 BA strain tumors (the 3 tumors used in Series VI included) were repeatedly inoculated into 12 mice of C_{57} strain, 3 of the C_3H strain and 7 of the A strain. Twenty-seven tumors (5 No. 30, 8 No. 35, 10 No. 48 and 4 No. 54) resulted. Fifteen of the tumors were in C_{57} strain mice, 7 in C_3H and 5 in A strain mice. Three of the tumors (Nos. 35, 48 and 54) were transplanted through 5 generations in mice of other strains. Undoubtedly tumor No. 30 would have been transplantable, but the hosts bearing this tumor died.

SERIES VIII. Implants of 5 BA strain tumors (selected because a marked neutrophilia developed in their hosts) were repeatedly inoculated into 7 mice of C_{57}, 7 of C_3H and 7 of A strain. Thirty one tumors resulted (12 No. 16, 9 No. 35, 7 No. 36, 3 No. 48; 7 in mice of C_{57} strain, 14 in mice of C_3H strain and 10 in mice of A strain). Sufficient mice of alien strains were available, so that every one of the tumors, except those in hosts that died unexpectedly, were transplanted. Tumors No. 16, No. 35, No. 36 and No. 48 were transplanted through at least five generations in mice of the C_{57}, C_3H and A strains.

During a lapse of some months after the eighth series of experiments was concluded a number of new tumors developed in dibenzanthracene inoculated mice. It was therefore decided to carry out two more series of experiments, using these tumors.

SERIES IX. Implants of 12 tumors (Nos. 130, 140, 144, 145, 146, 149, 151, 152, 153, 157, 158 and 164), originating in mice of the C_{57} strain, were repeatedly inoculated into 21 mice of alien strains; 7 of the BA strain, 7 of the C_3H strain and 7 of the A strain. During the course of 85 days each mouse received 19 inoculations. Ten tumors (1 No. 144, 1 No. 164, 1 No. 151, 2 No. 140, 2 No. 146 and 3 No. 153) grew in the mice of the C_3H strain, while mice of the A and the BA strains remained refractory.

Every one of these 10 tumors was transplanted into a normal mouse of the BA strain, one of the C_3H strain and one of the A strain. Four of the tumors (Nos. 140, 146, 151 and 153) were carried through five generations in the mice of the three strains.

SERIES X. In these experiments small pieces of five tumors from mice of the BA strain (Nos. 127, 133, 134, 136 and 142) were repeatedly implanted into 21 mice of alien strains: 7 C_{57}, 7 C_3H and 7 A strain. Eleven tumors grew in the repeatedly inoculated hosts; 8 of these were No. 134, 1 was No. 127, 1 was No. 133 and 1 No. 164. Each of these tumors was transplanted into normal mice: one of the C_{57}, one of the C_3H and one of the A strain; some of these tumors (No. 134) were successfully transplanted through 5 generations in mice of alien strains.

From these 10 series of experiments 17 induced dibenzanthracene tumors were obtained that were thereafter transplantable into mice of the A, BA, C_3H, C_{57} and certain other available strains.

Fourteen of these tumors were strain-specific tumors that became changed during their growth in the repeatedly inoculated hosts, so that they were there-

TRANSPLANTATION OF EXPERIMENTALLY OBTAINED NON-STRAIN SPECIFIC TUMORS INTO MICE OF ALIEN STRAINS[5]

Treated Host	Tumor No.	Transplanted into mice of strains											
		(2nd Gen.)			(3rd Gen.)			(4th Gen.)			(5th Gen.)		
		A	C_3H	C_{57}	A	C_3H	C_{57}	A	C_3H	C_{57}	A	C_3H	C_{57}
Series X.													
A[9]	134	0	0	0									
C_3H	134	+	+	0	+	+	+	+	+	0	+	+	+
C_3H	134	0	+	+	+	+	0	+	+	+	0	+	0
C_3H	134	+	+	0	0	+	+	0	+	0			
C_3H	134	+	+	+	+	+	0	+	+	+	+	+	+
C_3H	134	Died											
C_{57}	134	0	0	0									
C_{57}	134	+	+	+	0	0	0						
Series VIII.													
C_3H	36	+	+	+	0	0	0						
C_3H	36	+	+	+	+	0	0	+	+	0	+	+	+
C_3H	36	+	+	+	0	+	+	+	+	+	0	0	+
C_3H	36	0	+ +	+	0 +	+ +	0 +	0 +	0 +	0 +	+	+	+
C_3H	36	+	+	0	0	0	0						
C_{57}	36	+	0	0	0	0	0						
C_{57}	36	+	+	+	0	0	+	+	+	+	0	0	0

after transplantable into many strains of mice. The other three, however, proved to be non-strain-specific, as they grew in one or more of the normal mice of alien strains, inoculated as controls, to determine whether each tumor was strain specific at the time it was used in the series.

[5] The zero sign indicates that in the designated generation the transplant of the tumor failed to grow in the mouse of the strain under which the sign stands. The plus sign indicates that the transplant of the tumor grew into a tumor in the mouse of the strain under which it stands.

Many of the tumors that grew in hosts whose resistance had become broken down did not lose their strain specificity during growth in the treated hosts; for instance, the A strain tumors No. 6, No. 7 and No. 11 in Series I, the C_3H strain tumors No. 8 and No. 13 in Series IV, and the C_{57} strain tumors, No. 144 and No. 164 in Series IX. Also, while many of the tumors did lose their strain specificity during growth in treated hosts, they were by no means equally transplantable into mice of alien strains. This was the case with the 8 No. 134 tumors in Series X and the 8 No. 36 tumors in Series VIII shown in Chart 1. In some instances the tumors from mice of the same strain in the same generation were not equally transplantable; tumor No. 36 from a treated mouse of C_3H strain, transplanted into $2C_3H$ mice in the second generation, was successfully passed on through five generations from the one C_3H mouse but not from the other (See Chart 1).

Every one of the 31 tumors that grew in mice of Series VIII, except 4 of the No. 36, were transplanted through at least five generations in mice of alien strains. They were not all, however, transplantable to the same extent in mice of alien strains For instance 5 of the 8 No. 35 tumors were 100 per cent transplantable, while the other 3 failed to grow in one mouse in the second generation, 3 mice in the third generation and one mouse in the fifth generation.

A number of experimentally changed tumors that were transplanted through five generations in mice of alien strains were later carried in mice of only one alien strain, but they still retained their ability to grow in mice of other strains. Thus "A" strain tumor No. 20 in Series I after being carried for 20 generations in BA strain mice proved to be 100 per cent transplantable into mice of the A, the BA, the C_3H and the C_{57} strains.

Every one of the non-strain specific and experimentally changed sarcomata remained 100 per cent transplantable into mice of the strain in which the tumor originated. The naturally non-strain-specific tumors were only rarely transplantable in alien strain mice while many of the experimentally obtained non-strain-specific tumors were 100 per cent, others about 80 per cent transplantable into mice of other strains.[6]

SPONTANEOUS MAMMARY GLAND TUMORS

Eighty spontaneous mammary gland carcinomata were studied. Only 4 of them occurred in mice of the strains used in this laboratory. In earlier investigations Bittner (1929, 1933, 1935) found that mammary gland mouse tumors are transplantable into mice of the same strain, but not usually into mice of other strains. These 80 carcinomata likewise proved to be strain-specific. Seventy-six of the carcinoma-bearing mice were sent to us by Dr. H. B. Andervont.[7] They were an agouti strain supposedly C_3H, but not the same as our C_3H strain, for the tumors were not transplantable in mice of our C_3H strain.

[6] Owing to the shortage of mice of the A and the C_3H strains, we were persuaded to use certain hybrid mice. The results were so at variance with those obtained previously with the mice of pure inbred strains that the data as well as every one of the hybrid mice were discarded.

[7] Dr. H. B. Andervont, United States Public Health Service.

SERIES XI. In these experiments pieces of 22 of the spontaneous mammary gland carcinomata from agouti mice were grafted at 5-day intervals into 7 mice of A strain, 7 of BA, 7 of C_3H and 7 of C_{57} strain. The results were entirely negative in the mice of A, BA and C_{57} strains, but the 7 mice of the C_3H strain produced 12 tumors. None of these 12 tumors were transplantable in normal mice of the A, BA or C_{57} strains, but 5 of them were passed through 5 generations in mice of the C_3H strain, and when fixed and stained proved to be adeno-carcinomata, resembling the original spontaneous tumors.

An effort was made to increase the transplantability of these mammary gland tumors by repeatedly inoculating some of the tumor bearing agouti hosts with some of the spontaneous carcinomata from other agouti mice.

SERIES XII. Implants of 10 spontaneous carcinomata were grafted at 5-day intervals into 8 of the agouti mice, each of which already had from 1 to 3 spontaneous mammary gland tumors, 13 in all. Fourteen of the 80 grafts grew in these 8 mice, which had, in addition, 13 spontaneous tumors. Not one of these tumors proved to be transplantable into mice of the C_3H or other available strains.

In some later experiments, 10 spontaneous cracinomata from agouti mice were engrafted successively (one every 5 days) into 15 other agouti mice, each of which already had from 1 to 3 mammary gland tumors. Twenty-six of the grafts grew in the 15 mice, in addition to the 21 spontaneous mammary gland tumors present when the experiments were begun, and one mouse developed another spontaneous tumor during the course of the experiment. One of these tumors, and that a spontaneous one, proved to be transplantable through a number of generations in mice of the C_3H strain, but was not transplantable in mice of the A, BA or C_{57} strains. It could therefore not be considered as nonstrain specific.

IMMUNITY

The procedure used in our experiments did not bring about immunity to the growth of a dibenzanthracene induced tumor inoculated into mice of the same strain as that in which the tumor had been induced.

Experiments comparable to those made on carcinoma-bearing mice in Series XII were carried out in mice in which primary dibenzanthracene sarcomata were developing. These hosts were inoculated every 3 or 4 days with one of the primary sarcomata that had developed in other mice of the same strain. Every one of the grafts of the primary tumors grew so rapidly that some of the hosts died shortly after the third inoculation, and only one survived the fifth.

In some experiments a number of the straight-line tumors were successively grafted at 3-day intervals into mice of the same strain as the animals from which the tumors were taken. Every one of the grafts grew and the host became moribund before more than five tumors could be implanted. In other experiments one tumor was permitted to grow to a large size before the second tumor was inoculated, but even then the second tumor grew, provided the host lived long enough.

Mice in which tumors from an alien strain grew and later regressed were not resistant to the growth of a tumor transplanted from an individual of their own strain. In every one of the 11 series of mice repeatedly inoculated with tumors

from alien strains there was at least one animal that remained refractory to the growth of every one of the implanted tumors; yet not one of these treated mice proved to be resistant to the growth of a tumor from a mouse of its own strain.

A number of mice of BA strain were implanted with a considerable amount of finely divided mouse embryo skin. This tissue grew into rather large teratomata. Some of these mice were grafted with a BA strain sarcoma while the teratomata were large, others when the teratomata were regressing or had regressed. Not one of the mice proved to be resistant to the growth of the sarcoma.

Mice bearing spontaneous mammary gland tumors were not resistant to the development of additional mammary gland tumors. Hence, as many of these tumors were only moderately malignant, some of the female mice developed as many as five mammary gland tumors during the course of two months. The animals did, however, prove to be somewhat refractory to the growth of similar tumors grafted from mice of their own strain, as was shown in Series XII.

In many instances a carcinoma was implanted on one side of a mouse and a sarcoma on the other. Both tumors grew and were transplanted through many generations in this manner. Sections of the fixed and stained material showed that each tumor had retained its own characteristics, one being an adenocarcinoma and the other a large spindle-cell sarcoma.

CONCLUSIONS

Every one of the 200 dibenzanthracene tumors induced in mice of pure inbred strains (A, BA, C_3H and C_{57}) proved to be 100 per cent transplantable into mice of the strain in which the tumor arose.

Implants of one hundred and twenty-three of the dibenzanthracene tumors were made into mice of the other three strains. One hundred and fourteen of these tumors proved to be strain-specific, while the implants of nine of them grew in a normal mouse of one or more of the alien strains.

A number of mice that were repeatedly implanted with pieces of alien strain tumors to the growth of which they had previously proved refractory, lost their refractoriness to the growth of one or more of the implanted alien strain tumors.

Fourteen of the tumors that proved to be strain specific when implanted in normal young mice of alien strains lost their strain specificity when they grew in a repeatedly implanted host of an alien strain, so that they were thereafter transplantable not only into mice of their original strain and of the treated strain, but also into mice of many other strains.

The 80 spontaneous mammary gland tumors studied proved to be strain specific.

Repeated inoculation of mammary gland tumors into hosts of alien strains did not result in the loss of the tumors' strain specificity, although a number of them became transplantable into mice of a closely related (C_3H) strain.

Mice bearing spontaneous mammary gland tumors were to a great extent refractory to similar grafted tumors from other mice of the same strain, but

were not immune to the development of additional spontaneous mammary gland tumors.

No immunity to the growth of a dibenzanthracene tumor could be demonstrated in mice of the strain in which the tumor arose, not even after the mice had remained resistant to the growth of many tumors grafted from mice of another strain. Mice in which one primary induced tumor was developing were not refractory to the growth of grafts of induced tumors developing in other mice of the same strain.

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STUDIES ON DENTAL CARIES

II. THE USE OF THE NORMAL PROBABILITY CURVE FOR EXPRESSING THE AGE DISTRIBUTION OF ERUPTION OF THE PERMANENT TEETH

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INTRODUCTION

In approaching the problem of dental caries from a public health point of view, it is desirable to include a survey of possible methods by means of which some of the well recognized complexities of the problem may be simplified. A first step in simplification would appear to involve the application of those techniques which have been found useful in the study of other diseases. In this connection statistical methods, particularly those which have been found applicable to epidemiological problems, offer much promise. This perspective leads to the consideration that data on the eruption of teeth may have some bearing on the problem of dental caries since attack of the teeth by caries may be a function of the length of time the teeth are exposed to the environment of the mouth. A survey of methods for determining and expressing data on the eruption of the several teeth, is therefore, indicated. The purpose of the present paper is to examine those methods which previously have been used for expressing data on tooth eruption and to describe one by means of which the age distribution of eruption is expressed accurately and quantitatively by the normal (Gaussian) frequency function.

METHODS OF EXPRESSING TIME OF ERUPTION OF THE TEETH

Students of the problem of tooth eruption, in general, have used, for describing the eruption process, expressions which have been concerned largely with calculations of the median and average chronological ages at which the several permanent and deciduous teeth erupt.

Suk (1) determined, for Zulu boys and girls, the percentage with each permanent tooth erupted and appearing at each age and calculated the mean ages of eruption of the several teeth. In order to determine the variation or range of the eruption period, this worker noted, also, the earliest and latest ages at which eruption was observed in different individuals. Bean (2) expressed the time of eruption in terms of the average number of specified teeth erupted or erupting at each age. He also determined the median, the mode and the extremes of eruption dates for specific teeth. Magitot (3), Broca (4), Mayet (5), Cherot (6), Welcker (7), and Livy (8), each expressed tooth eruption on the basis of that age at which fifty per cent of the individuals under observation had a particular tooth in the mouth, so obtaining the median ages

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of eruption. Boas (9) calculated percentages of permanent teeth present in the upper jaws of Puerto Rican, Boston, New York, and Saxony boys and compared the per cent of permanent teeth present in the different age and locality groups. In another study, on the eruption of deciduous teeth in Hebrew infants (10), Boas calculated the averages and standard deviations of the age distributions of eruption of the several teeth in boys and girls. Cattell (11) determined the median ages and the lower and upper quartile ranges of the age distribution of tooth eruption in 7,689 children using ogives which were constructed by connecting the points designating observed percentages of children having specified teeth present at various ages. In addition, this worker determined the median number of all permanent teeth erupted at successive chronological ages from 5.5 to 15 years. Cohen (12) also has constructed ogives giving the percentages of children of various ages having specified permanent teeth erupted and from these curves has calculated the median ages and the quartile ranges of the distributions for the several teeth. The percentages of teeth erupting during yearly age intervals were obtained by differencing consecutive points on the ogive curves. None of the above studies contain provision, however, for a full description of the eruption process by means of a mathematical function A group of English investigators, (13) on the other hand, have fitted logistic curves of the form,

$$Y = \frac{K}{1 + e^{a+bz}}$$

to data on the percentage distribution of single teeth erupted at different ages. These curves were considered to describe the data satisfactorily and were used to obtain the ages at which fifty per cent of the children examined had specified permanent teeth erupted.

THE APPLICABILITY OF THE NORMAL PROBABILITY CURVE FOR DESCRIBING THE AGE DISTRIBUTION OF ERUPTION OF THE PERMANENT TEETH

As an integral part of a study of the characteristics of dental caries in 4,416 white school children between the ages of 6 and 15 years, it was found necessary to develop a quantitative expression which would serve to describe accurately and completely the age distribution of eruption of the permanent teeth. The data of the study consist of observations of the numbers of the different permanent teeth which had erupted[2] in the mouths of different[3] children at successive chronological ages. For purposes of analysis it was found convenient to express these data in terms of percentage distributions; that is, to obtain for each type of tooth the distribution of the per cent of children in yearly age groups in whom the tooth had erupted. When these percentage age

[2] In the present study a tooth was considered erupted if one millimeter or more was seen above the gum. Extracted permanent teeth were counted as having erupted.

[3] In using data consisting of observations on *different* children at different ages it is necessary to assume that the population at each age is representative of the population as a whole. This assumption is commonly made in many growth studies and would appear to be valid in this work.

distributions are plotted on arithmetic graph paper, they follow the characteristic S-shaped ogive which has been used for expressing eruption data. Thus, for any particular type of tooth the percentages of children having this particular tooth erupted increase with age; at relatively early ages, the percentages are small and the increase with advancing age is slow; at later chronological ages, when the per cent of children having the tooth erupted is approaching 50, the increase with age becomes rapid; at older ages, when the proportion of the children having the tooth in the mouth approaches 100 per cent, the change with age again becomes slow. The relative regularity of this progressive age change in the per cent of children having any specified perma-

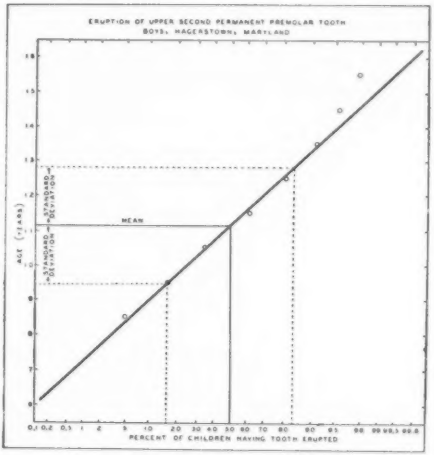
ERUPTION OF UPPER SECOND PERMANENT PREMOLAR TOOTH
BOYS, HAGERSTOWN, MARYLAND
MEAN
PERCENT OF CHILDREN HAVING TOOTH ERUPTED

FIG. 1

nent tooth erupted suggests that the process of eruption might be satisfactorily described by a simple mathematical equation. The group of English workers quoted above (13) have used the logistic equation to describe this procession, while a number of other workers, mentioned previously, have used various constants of the normal frequency function to describe certain limited characteristics of the progression. The mathematical character of both the logistic and normal curves is such that it is reasonable to suppose that either may be used satisfactorily for expressing the age distribution of eruption of the teeth. However, as will become apparent in this and subsequent papers, a number of useful and practical results are possible through the full and systematic use of the equation of the normal curve as a mathematical description of the eruption process.

In the course of the analysis of eruption data, it was found that the percentage distribution of eruption of each of the permanent teeth appear as fairly regular straight lines when the data for each tooth are plotted on arithmetic

probability paper. The absence of marked variation of the observed percentages from the linear trends on probability paper, makes readily possible the drawing of "free-hand" straight lines through the plotted data. From these lines, for each type of tooth, the means and standard deviations of the normal curves may be read directly with considerable accuracy.[4] An example of this procedure is given in Figure 1 which shows the observed percentages and the straight line graph on probability paper for the corresponding upper second pre-molar teeth (right and left),[5] as observed in approximately 2,200 Hagerstown, Maryland, school boys. From the straight line drawn on the graph it may be noted that the mean, median and mode of the age distribution of eruption of this tooth fall at 11.18 years of age. The standard deviation of the normal frequency curve representing this distribution may be read as equal to 1.68 years. It may

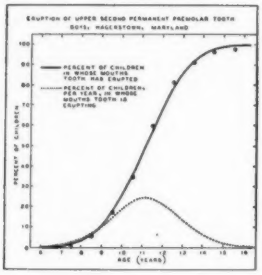
ERUPTION OF UPPER SECOND PERMANENT PREMOLAR TOOTH
BOYS, HAGERSTOWN, MARYLAND
PERCENT OF CHILDREN IN WHOSE MOUTHS TOOTH HAS ERUPTED
PERCENT OF CHILDREN PER YEAR, IN WHOSE MOUTHS TOOTH IS ERUPTING
PERCENT OF CHILDREN
AGE (YEARS)

FIG. 2

be observed also that the quartile and percentile ranges and other characteristics of the distribution are readily obtainable from this graph.

Further, through the use of the normal probability function, it is possible to take advantage of the extensive knowledge now available regarding the statistical characteristics of this function. Useful expressions for eruption data become available in the form given in Figure 2 which shows both the normal and cumulated percentage frequency distribution of eruption for the corresponding upper second pre-molar teeth. From such graphic presentations, or directly from any of the well-known tables of the normal probability function, it is possible to obtain other pertinent and meaningful data. Thus, the area under the normal eruption curve below any point on the age scale gives the expected, theoretical, value of the percentage of children below that age in whose mouths the tooth has erupted. The ordinate of the cumulated frequency curve at the same point on the age scale gives the same value. The area of the normal curve

[4] See, Reed, L. J.: Statistical Treatment of Biological Problems. Chapter VI, Biological Effects of Radiation. McGraw-Hill Book Co., New York (1936).

[5] Data, combined as averages for teeth in corresponding positions on the right and left sides of the body, are given in the text and in the figures throughout the present report. This procedure is considered justifiable since there is no evidence of a difference in time of eruption for such corresponding teeth (1) (11) (12), and since no statistically significant differences could be demonstrated in the present data for either the means or standard deviations of the frequency distributions for corresponding teeth.

TABLE 1

PERCENT OF CHILDREN HAVING THE SPECIFIED CORRESPONDING PERMANENT TEETH ERUPTED. 4,416 BOYS AND GIRLS, HAGERSTOWN, MARYLAND

Age (Years) Last Birthday[1]		6		7		8		9		10		11		12		13		14		15	
No. Cases		171	156	197	205	231	256	253	240	270	259	262	269	299	297	267	278	199	165	83	58
Sex		Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
Percent of Cases Having Designated Teeth Erupted																					
Upper Jaw Right & Left Teeth	Central Incisor	14.9	26.3	54.3	70.1	90.0	94.1	98.4	99.6	100.0	99.6	100.0	100.0	100.0	99.8	100.0	100.0	100.0	—	100.0	100.0
	Lateral Incisor	2.6	3.5	15.2	22.3	43.5	63.5	80.2	93.7	95.9	94.2	96.7	98.1	98.7	98.5	99.4	98.7	98.7	98.8	97.6	96.7
	Canine	—	1.3	0.8	0.7	1.3	2.1	5.3	8.5	15.6	35.1	40.8	66.4	69.4	84.8	90.6	93.7	93.2	95.8	96.4	98.3
	First Pre-Molar	—	0.6	1.3	2.2	10.0	10.6	27.1	36.5	50.6	62.5	76.5	87.5	90.6	95.1	97.4	98.6	99.0	99.4	100.0	99.2
	Second Pre-Molar	0.3	0.6	0.2	1.2	4.8	4.5	16.0	18.7	33.5	44.6	60.9	70.8	80.4	83.8	90.8	94.1	95.2	98.5	97.6	97.5
	First Molar	54.7	61.2	88.8	93.0	97.0	99.6	100.0	99.6	100.0	100.0	100.0	100.0	100.0	99.8	100.0	99.8	100.0	100.0	100.0	100.0
	Second Molar	—	—	—	—	—	—	0.4	0.4	2.6	6.0	17.0	25.6	45.5	57.4	73.2	79.9	84.7	90.6	87.9	91.7
Lower Jaw Right & Left Teeth	Central Incisor	63.4	79.8	91.4	94.7	99.4	99.2	99.0	99.6	99.6	100.0	100.0	100.0	100.0	100.0	99.6	100.0	100.0	100.0	100.0	100.0
	Lateral Incisor	12.3	22.4	45.7	61.2	85.7	92.6	96.6	99.6	98.7	100.0	99.8	100.0	99.8	100.0	99.8	100.0	100.0	100.0	100.0	100.0
	Canine	0.4	1.3	0.5	1.2	1.7	7.4	12.1	41.0	45.0	70.3	76.5	93.9	91.0	96.8	98.5	99.8	98.7	99.4	100.0	100.0
	First Pre-Molar	0.3	0.6	—	1.5	2.8	5.9	16.6	32.5	42.8	55.6	72.9	86.2	85.1	94.3	96.8	98.0	98.2	99.1	98.8	100.0
	Second Pre-Molar	—	1.0	—	0.7	3.0	4.3	9.7	17.5	29.6	36.1	53.8	66.1	72.2	79.6	84.3	89.2	92.7	97.6	93.4	95.0
	First Molar	65.5	76.9	91.9	93.7	98.9	99.7	100.0	100.0	100.0	100.0	99.6	99.6	99.8	100.0	99.8	100.0	99.5	100.0	100.0	100.0
	Second Molar	—	—	—	—	—	—	0.8	1.7	7.6	14.5	30.1	43.1	58.0	70.9	84.8	83.4	87.7	93.6	95.8	96.7

[1] The average age of the children included in each of the separate age groups falls approximately ½ year above the age as given in the table except for the 6 year old group whose average age is at approximately 6¾ years. This fact was considered throughout the analysis of the data.

between any two points on the age scale gives the proportion of the teeth which erupt during the age interval represented between these two points. The derivative of the cumulated curve, or the value of the ordinate of the normal curve, at any point on the age scale, gives the instantaneous rate or the velocity of eruption. In addition other useful data may be derived from this curve.[6]

It may be emphasized at this point that there are no inherent reasons for using exclusively the normal probability curve for expressing the eruption of the permanent teeth. However, the considerations which favor the use of the normal curve are that this curve appears to fit the eruption data very satisfactorily, the use of probability paper and of the normal curve is well-known, and, finally, a number of advantages may be expected to accrue in connection with the further application of the normal probability theory to data on tooth eruption.

AN ILLUSTRATION OF THE USE OF THE NORMAL CURVE FOR DESCRIBING ERUPTION OF THE PERMANENT TEETH

Considerations, as presented above, of the advantages to be derived through the use of the Gaussian function in describing the tooth eruption process, have

TABLE 2

MEANS AND STANDARD DEVIATIONS OF AGE DISTRIBUTION OF ERUPTION OF CORRESPONDING PERMANENT TEETH. 4,416 BOYS AND GIRLS, HAGERSTOWN, MARYLAND

Teeth	Upper Jaw (Corresponding Teeth)				Lower Jaw (Corresponding Teeth)			
	Mean (years)		Stand. Dev. (years)		Mean (years)		Stand. Dev. (years)	
	Boys	Girls	Boys	Girls	Boys	Girls	Boys	Girls
Central Incisor	7.49	7.20	0.75	0.75	6.50	6.19	0.74	0.70
Lateral Incisor	8.62	8.15	1.10	0.94	7.64	7.31	0.80	0.75
Canine	11.80	11.05	1.42	1.37	10.70	9.85	1.15	1.15
First Pre-molar	10.42	10.00	1.50	1.40	10.75	10.20	1.42	1.41
Second Pre-molar	11.18	10.82	1.68	1.60	11.45	11.00	1.77	1.70
First Molar	6.64	6.54	0.75	0.70	6.44	6.12	0.75	0.87
Second Molar	12.70	12.40	1.18	1.40	12.20	11.90	1.20	1.55

led to the fitting of the normal curve to data on the eruption of the permanent teeth obtained from a survey of 4,416 Hagerstown, Maryland, school children. The data are given in detail in Tables 1 and 2. In Table 1 the percentages of white boys and girls having particular teeth erupted are given for each of the separate permanent teeth (except the third molar) for each single year of age[7] from 6 through 15 years. These percentages, derived from direct observation, were first plotted on arithmetic probability paper and straight lines were drawn, by inspection, through the plotted points. The straight lines so obtained are illustrated in Figure 3 for the Hagerstown boys. Means and standard devia-

[6] One of the most important extensions of this analysis of eruption data, which will be presented in a following paper, consists in the derivation of accumulated years of exposure of specified teeth in the mouth (post-eruptive tooth years of exposure) which is obtained by integration of the cumulated frequency curve given in Figure 2.

[7] Age at last birthday.

tions of the normal distributions for each permanent tooth were read from such graphs on probability paper. These means and standard deviations for the distribution of each of the 14 corresponding pairs of permanent teeth are assembled in Table 2.[8] From the data given in this table it is possible to describe precisely, and with considerable completeness, the eruption of the permanent teeth of the children examined.

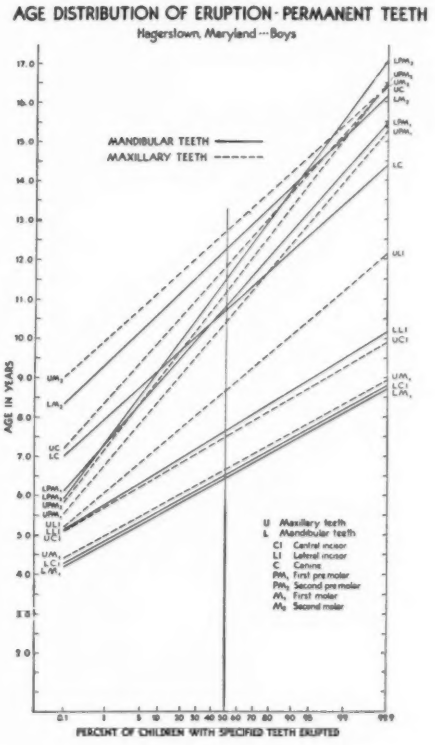

FIG. 3

DISCUSSION

It is apparent from the foregoing sections that a simple mathematic equation, the normal probability function, may be used to describe the eruption of the permanent teeth in the human mouth. This fact is of considerable importance in the quantitative approach to a variety of problems related to growth and development, and dental disease. The description of the eruption of the teeth by means of a mathematical equation such as the normal curve, assists measurably, also, in the development of a variety of practical applications of eruption data.

[8] See footnote 5.

The first of these developments is concerned with the derivation of a complete description of the age distribution of eruption of a tooth from data which do not cover the full range of ages required for the eruption process. In this problem, as in many others, the finding that a particular type of mathematical function will fit observed data, allows an estimation of the complete progression in those instances where sufficient observations are available to permit the calculation of the parameters of the function. The assumption that the normal probability curve satisfactorily fits the age distribution of eruption of the permanent teeth makes possible, therefore, the estimation of the complete eruption process in situations where the observed data may be somewhat limited. Since the normal curve, as used in this problem, requires the determination of only two arbitrary parameters (the mean and standard deviation) it is possible to estimate the full age distribution of eruption from two observed points on the curve.[9] Usually, it would not be desirable to obtain the parameters of the normal curve from two points alone. On the other hand, the possibility of this procedure illustrates the utility of the finding that the normal probability function appears to fit satisfactorily the age distribution of tooth eruption.[10]

On the basis of such analysis as presented, it is possible, in addition, to determine readily whether or not the eruption time for any of the permanent teeth is different among different population groups. For example, the proportion of the several permanent teeth erupted at any age may be read directly from charts on probability paper such as is illustrated in Figure 3. The proportions or percentages obtained from such charts may be compared with similar proportions of teeth found erupted in other child population.[11]

Another simple use which may be made of the eruption curves concerns the determination of the average number of all erupted permanent teeth to be found in children at any specified age. This value may be obtained by averaging[12] the several proportions of children having *specified* teeth erupted at any given age as read directly from probability paper charts, similar to that given

[9] The reliability of any single observed point, or percentage of children having a specified tooth erupted, depends on two factors—the number of children observed and the value of the percentage itself. According to an elementary theorem of probability, the sampling variation which may be assigned to an observed proportion decreases as the proportion approaches the value one-half. It is apparent, therefore, that estimates of the age distribution are most reliable when based on percentages from the middle range of the distribution.

[10] In this connection it is of interest to point out that the normal curve appears to fit the observational data on eruption which has been published by a number of other investigators (1) (2) (6) (9) (10) (11) (12) (13).

[11] The data presented in Table 2, from Hagerstown, Maryland, furnish one standard for native-born white children which may be used for comparison with similar data for children in other localities. These data constitute a fairly large series of observations which may be considered representative of a typical small urban community in the eastern section of this country. Hagerstown is a city of approximately 30,000 population. Approximately 94 percent of the children attending the first 8 grades in the public schools were examined. Over 98 percent of them are native-born, about 90 percent having native-born parents.

[12] The average obtained in this manner is equivalent to an average found from counts of the total number of permanent teeth in the mouths of children of specified age since both averages are assumed to be derived from the same number of individuals.

in Figure 3. Averages so obtained, for different geographic localities, race and sex groups, may then be assembled and compared for the purpose of expressing differences in the process of eruption in the different groups.[13] For purposes of illustrating differences between the two sexes in the total number of permanent teeth in the mouths at successive specified chronological ages, the average number of teeth erupted was determined for the survey data reported in this paper. Table 3 gives the basic data derived by averaging the percentages of

TABLE 3

AVERAGE PERCENT AND AVERAGE NUMBER OF ALL PERMANENT TEETH (EXCEPTING THIRD MOLARS) ERUPTED INTO THE MOUTH AT SPECIFIED CHRONOLOGICAL AGES. 4,416 BOYS AND GIRLS, HAGERSTOWN, MARYLAND

Age (Years)	Upper Jaw				Lower Jaw				Both Jaws				Difference: Girls Minus Boys	
	Boys		Girls		Boys		Girls		Boys		Girls			
	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number
4.5	0.0	0.0	0.0	0.0	0.1	0.0	0.6	0.1	0.1	0.0	0.3	0.1	0.2	0.1
5.5	1.0	0.1	1.2	0.2	2.8	0.4	5.8	0.8	1.9	0.5	3.5	1.0	1.6	0.5
6.5	7.9	1.1	10.0	1.4	15.9	2.2	21.3	3.0	11.9	3.3	15.7	4.4	3.8	1.1
7.5	22.5	3.1	26.8	3.8	32.7	4.6	36.9	5.2	27.6	7.7	31.9	8.9	4.3	1.2
8.5	36.1	5.1	40.7	5.7	42.7	6.0	46.6	6.5	39.4	11.0	43.6	12.2	4.2	1.2
9.5	46.7	6.5	51.9	7.3	49.7	7.0	56.3	7.9	48.2	13.5	54.1	15.1	5.9	1.6
10.5	57.6	8.1	64.1	9.0	60.5	8.5	69.6	9.7	59.0	16.5	66.8	18.7	7.8	2.2
11.5	70.1	9.8	77.4	10.8	75.0	10.5	82.2	11.5	72.6	20.3	79.8	22.3	7.2	2.0
12.5	83.2	11.6	88.6	12.4	87.9	12.3	91.4	12.8	85.6	24.0	90.0	25.2	4.4	1.2
13.5	93.3	13.1	95.7	13.4	95.8	13.4	96.7	13.5	94.5	26.5	96.2	26.9	1.7	0.4
14.5	98.3	13.8	98.8	13.8	98.9	13.9	99.0	13.9	98.6	27.6	98.9	27.7	0.3	0.1
15.5	99.7	14.0	99.8	14.0	99.8	14.0	99.8	14.0	99.8	27.9	99.8	27.9	0.0	0.0
16.5	100.0	14.0	100.0	14.0	100.0	14.0	100.0	14.0	100.0	28.0	100.0	28.0	0.0	0.0

Girls and Boys—Average difference in number of permanent teeth..... 0.9

children having the separate permanent teeth erupted in the mouth together with the calculated average number of permanent teeth erupted for the complete age range of the development of the permanent dentition. These averages are given separately for the upper and lower jaws and for both jaws together. It is of interest to note that, during the major part of the age range of the development of the permanent dentition, girls have an average of 0.9 more permanent teeth present in the mouth than boys, the maximum difference between the sexes occurring in the age interval 10 to 11 years when girls have 2.2 more permanent teeth than boys.

The final point to be considered in this discussion will indicate the implications of these results in the special problem of dental caries. The very practical significance of the development of a precise method for describing the eruption of the teeth becomes apparent when it is recognized that attack by caries is dependent (unpublished findings) on length of exposure of the teeth in the oral cavity and therefore directly dependent on time of eruption. The method presented in this paper for describing the age distribution of eruption facilitates the calculation of the time of exposure of the teeth in the mouth.

[13] As an illustration of an additional application of eruption data, it may be pointed out that Cattell (11) has used the *median* number of permanent teeth found in children as an index of maturity.

SUMMARY

1. A method is presented by means of which the age distribution of tooth eruption may be accurately described by the normal probability function.

2. A description of the age distribution of eruption of the permanent teeth in 4,416 school children is presented.

3. The significance of the application of the normal curve in the description of tooth eruption is discussed.

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